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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/083,058	02/25/2002	Svend Havelund	5386.224-US	6987

7590 05/27/2004

Reza Green, Esq.
Novo Nordisk of North America, Inc.
Suite 6400
405 Lexington Avenue
New York, NY 10174-6401

EXAMINER	
GUPTA, ANISH	

ART UNIT	PAPER NUMBER
1654	

DATE MAILED: 05/27/2004

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/083,058	HAVELUND ET AL.	
	Examiner	Art Unit	
	Anish Gupta	1654	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☐ Claim(s) *61-69* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☐ Claim(s) *61-69* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

The preliminary amendment filed 2-25-02 is acknowledged. Claims 1-60 were cancelled and claims 61-69 were added. Claims 61-69 are pending in this application.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.
>
> (e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or on an international application by another who has fulfilled the requirements of paragraphs (1), (2), and (4) of section 371(c) of this title before the invention thereof by the applicant for patent.

The changes made to 35 U.S.C. 102(e) by the American Inventors Protection Act of 1999 (AIPA) and the Intellectual Property and High Technology Technical Amendments Act of 2002 do not apply when the reference is a U.S. patent resulting directly or indirectly from an international application filed before November 29, 2000. Therefore, the prior art date of the reference is determined under 35 U.S.C. 102(e) prior to the amendment by the AIPA (pre-AIPA 35 U.S.C. 102(e)).

1. Claims 61-69 are rejected under 35 U.S.C. 102(e) as being anticipated by Havelund et al.

The claims are drawn to water soluble aggregate of an insulin derivative having a lipophilic group and has a molecular weight larger than aldolase and comprise at lease 2 zinc ions per 6 moles of insulin derivative.

The reference teach a composition comprising 600 nmol/ml of Lys B29-Nε-(hexadecanoyl)-insulin, 7 mM of sodium phosphate buffer at pH 7.5, 10 mM sodium chloride, 16 mM phenol, 16 mM cresol, 2-3 Zn+2/hexamer and 1.6%(w/v) glycerol (see col. 32, lines 34-48). The reference also discloses similar pharmaceutical formulations for Lys B29-Nε lithocholyl human insulin (see col. 31 and 32, lines 54-67 and 19-31). Note that this composition is similar to the composition as disclosed in the specification on page 13, line 28-29. Therefore, since the reference discloses the same composition as disclosed in the specification, with the same ionic strength and pH, the composition described in Havelund et al. would inherently result in aggregate formations.

2. Claims 61-69 are rejected under 35 U.S.C. 102(e) as being anticipated by Norup et al.

The claims are drawn to water soluble aggregate of an insulin derivative having a lipophilic group and has a molecular weight larger than aldolase and comprise at lease 2 zinc ions per 6 moles of insulin derivative.

The reference teaches various insulin formulations that comprise insulin, phenolic compound such as cresol, glycerol, sodium chloride and varying amounts of zinc (see col. 4, lines 1-16 and 31-61). The pH of the composition is of the 7.2 when 20 mM of NaCl is present (see col. 4, lines 34-62). The composition utilizes insulin derivatives that include, B29-Nε-(N-lithocholyl -γ-glutamyl)-des(B30)-human insulin (see col. 3, lines 499-61). The difference between the prior art and the instant application is that the reference does not specifically teach aggregation of the insulin. However, since the reference discloses a composition with similar ionic strength and pH as the claimed composition, the composition disclosed by the reference would necessarily result in aggregate formations. Moreover, since the reference teaches pharmaceutical formulations that are

intended to be used in-vivo, the formations aggregates would have occurred after injections since the environment would have the ionic strength and pH necessary for aggregates to form. Note the claims state that the aggregates are "formed in an environment having an ionic strength and pH of the tissue **after** subcutaneous injections."

3. Claims 61-69 are rejected under 35 U.S.C. 102(b) as being unpatentable over Havelund et al. (WO 95/07931).

The claims are drawn to water soluble aggregate of an insulin derivative having a lipophilic group and has a molecular weight larger than aldolase and comprise at lease 2 zinc ions per 6 moles of insulin derivative.

The reference teach insulin composition comprising 600 nmol/ml of insulin, 7 mM of sodium phosphate buffer at pH 7.5, 10 mM sodium chloride, 16 mM phenol, 16 mM cresol, 2-3 Zn+2/hexamer and 1.6%(w/v) glycerol (see page 55-56). For insulin analogs, the reference teaches, as acknowledge by Applicants on page 10 of the specification, the use of NεB29-lithocholoyl-α-glutamyl des (B30) (see page 54, lines 13-25). Furthermore, the reference states that the parenteral administration may be performed by subcutaneous injection (see page 27, lines 8-10). Therefore, since the reference discloses the same composition as disclosed in the specification, with the same ionic strength and pH, the composition described in Havelund et al. would necessarily result in aggregate formations. Thus since all of the structural limitation of the compound are met and the same mode of administration is also disclosed, the aggregation of the compound, after injection, would be necessarily be achieved.

4. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Anish Gupta whose telephone number is (571)272-0965. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Brenda Brumback , can normally be reached on (571) 272-0961. The fax phone number of this group is (703) 308-4242.

Any inquiry of a general nature or relating to the status of this application should be directed to the Group receptionist whose telephone number is (703) 308-0196.

5/18/04

Anish Gupta
Patent Examiner

Notice of References Cited	Application/Control No. 10/083,058	Applicant(s)/Patent Under Reexamination HAVELUND ET AL.	
	Examiner Anish Gupta	Art Unit 1654	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-5,866,538	02-1999	Norup et al.	514/3
	B	US-6,011,007	01-2000	Havelund et al.	514/3
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N	WO95/07931	03-1995	PCT	Havelund et al.	----
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	
	W	
	X	5/19/04

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(71) Applicant *(for all designated States except US)*: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: HAVELUND, Svend [DK/DK]; Kurvej 24, DK-2880 Bagsvaerd (DK). HALSTRØM, John, Broberg [DK/DK]; Søndergade 44, DK-3390 Hundested (DK). JONASSEN, Ib [DK/DK]; Valby Langgade 10, DK-2500 Valby (DK). ANDERSEN, Asser, Sloth [DK/DK]; Grundtvigsvej 35, 2.tv., DK-1864 Frederiksberg C (DK). MARKUSSEN, Jan [DK/DK]; Kikudbakken 7, DK-2730 Herlev (DK).

(74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsvaerd (DK).

(54) Title: ACYLATED INSULIN

(57) Abstract

The present invention relates to protracted human insulin derivatives in which the A21 and the B3 amino acid residues are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; Phe^{B1} may be deleted; the B30 amino acid residue is a) a non-codable, lipophilic amino acid having from 10 to 24 carbon atoms, in which case an acyl group of a carboxylic acid with up to 5 carbon atoms is bound to the ϵ-amino group of Lys^{B29}; or b) the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys, in any of which cases the ϵ-amino group of Lys^{B29} has a lipophilic substituent; and any Zn^{2+} complexes thereof with the proviso that when B30 is Thr or Ala and A21 and B3 are both Asn, and Phe^{B1} is present, then the insulin derivative is always present as a Zn^{2+} complex.

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ACYLATED INSULIN

FIELD OF THE INVENTION

The present invention relates to novel human insulin derivatives which are soluble and have a protracted profile of action, to a method of providing such derivatives, to pharmaceutical compositions containing them, and to the use of such insulin derivatives in the treatment of diabetes.

BACKGROUND OF THE INVENTION

Many diabetic patients are treated with multiple daily insulin injections in a regimen comprising one or two daily injections of a protracted insulin to cover the basal requirement supplemented by bolus injections of a rapid acting insulin to cover the requirement related to meals.

Protracted insulin compositions are well known in the art. Thus, one main type of protracted insulin compositions comprises injectable aqueous suspensions of insulin crystals or amorphous insulin. In these compositions, the insulin compounds utilized typically are protamine insulin, zinc insulin or protamine zinc insulin.

Certain drawbacks are associated with the use of insulin suspensions. Thus, in order to secure an accurate dosing, the insulin particles must be suspended homogeneously by gentle shaking before a defined volume of the suspension is withdrawn from a vial or expelled from a cartridge. Also, for the storage of insulin suspensions, the temperature must be kept within more narrow limits than for insulin solutions in order to avoid lump formation or coagulation.

While it was earlier believed that protamines were non-immunogenic, it has now turned out that protamines can be

immunogenic in man and that their use for medical purposes may lead to formation of antibodies (Samuel et al., Studies on the immunogenecity of protamines in humans and experimental animals by means of a micro-complement fixation test, Clin. Exp. Immunol. 33, pp. 252-260 (1978)).

Also, evidence has been found that the protamine-insulin complex is itself immunogenic (Kurtz et al., Circulating IgG antibody to protamine in patients treated with protamine-insulins. Diabetologica 25, pp. 322-324 (1983)). Therefore, with some patients the use of protracted insulin compositions containing protamines must be avoided.

Another type of protracted insulin compositions are solutions having a pH value below physiological pH from which the insulin will precipitate because of the rise in the pH value when the solution is injected. A drawback with these solutions is that the particle size distribution of the precipitate formed in the tissue on injection, and thus the timing of the medication, depends on the blood flow at the injection site and other parameters in a somewhat unpredictable manner. A further drawback is that the solid particles of the insulin may act as a local irritant causing inflammation of the tissue at the site of injection.

WO 91/12817 (Novo Nordisk A/S) discloses protracted, soluble insulin compositions comprising insulin complexes of cobalt(III). The protraction of these complexes is only intermediate and the bioavailability is reduced.

Human insulin has three primary amino groups: the N-terminal group of the A-chain and of the B-chain and the ϵ-amino group of Lys^{B29}. Several insulin derivatives which are substituted in one or more of these groups are known in the prior art. Thus, US Patent No. 3,528,960 (Eli Lilly) relates to N-carboxyaroyl insulins in which one, two or three primary amino groups of the

insulin molecule has a carboxyaroyl group. No specifically $N^{\epsilon B29}$-substituted insulins are disclosed.

According to GB Patent No. 1.492.997 (Nat. Res. Dev. Corp.), it has been found that insulin with a carbamyl substitution at $N^{\epsilon B29}$ has an improved profile of hypoglycaemic effect.

JP laid-open patent application No. 1-254699 (Kodama Co., Ltd.) discloses insulin wherein a fatty acid is bound to the amino group of Phe^{B1} or to the ϵ-amino group of Lys^{B29} or to both of these. The stated purpose of the derivatisation is to obtain a pharmacologically acceptable, stable insulin preparation.

Insulins, which in the B30 position have an amino acid having at least five carbon atoms which cannot necessarily be coded for by a triplet of nucleotides, are described in JP laid-open patent application No. 57-067548 (Shionogi). The insulin analogues are claimed to be useful in the treatment of diabetes mellitus, particularly in patients who are insulin resistant due to generation of bovine or swine insulin antibodies.

By "insulin derivative" as used herein is meant a compound having a molecular structure similar to that of human insulin including the disulfide bridges between Cys^{A7} and Cys^{B7} and between Cys^{A20} and Cys^{B19} and an internal disulfide bridge between Cys^{A6} and Cys^{A11}, and which have insulin activity.

However, there still is a need for protracted injectable insulin compositions which are solutions and contain insulins which stay in solution after injection and possess minimal inflammatory and immunogenic properties.

One object of the present invention is to provide human insulin derivatives, with a protracted profile of action, which are soluble at physiological pH values.

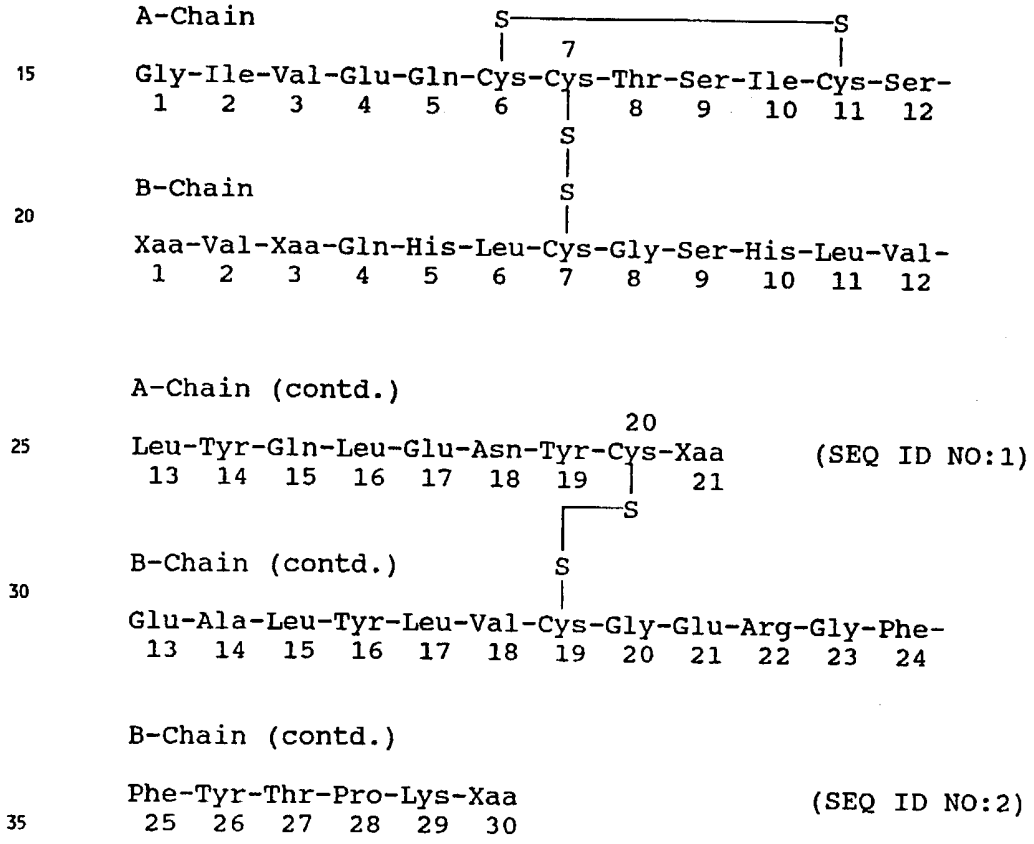
Another object of the present invention is to provide a pharmaceutical composition comprising the human insulin derivatives according to the invention.

It is a further object of the invention to provide a method of making the human insulin derivatives of the invention.

SUMMARY OF THE INVENTION

Surprisingly, it has turned out that certain human insulin derivatives, wherein the ϵ-amino group of Lys^{B29} has a lipophilic substituent, have a protracted profile of action and are soluble at physiological pH values.

Accordingly, in its broadest aspect, the present invention relates to an insulin derivative having the following sequence:



wherein

Xaa at positions A21 and B3 are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys;

Xaa at position B1 is Phe or is deleted;

Xaa at position B30 is (a) a non-codable, lipophilic amino acid having from 10 to 24 carbon atoms, in which case an acyl group of a carboxylic acid with up to 5 carbon atoms is bound to the ϵ-amino group of Lys^{B29}, (b) any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys, in which case the ϵ-amino group of Lys^{B29} has a lipophilic substituent or (c) deleted, in which case the ϵ-amino group of Lys^{B29} has a lipophilic substituent; and any Zn^{2+} complexes thereof,provided that when Xaa at position B30 is Thr or Ala, Xaa at positions A21 and B3 are both Asn, and Xaa at position B1 is Phe, then the insulin derivative is a Zn^{2+} complex.

In one preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; the A21 and the B3 amino acid residues are, independently, any amino acid residues which can be coded for by the genetic code except Lys, Arg and Cys; Phe^{B1} may be deleted; the ϵ-amino group of Lys^{B29} has a lipophilic substituent which comprises at least 6 carbon atoms; and 2-4 Zn^{2+} ions may be bound to each insulin hexamer with the proviso that when B30 is Thr or Ala and A21 and B3 are both Asn, and Phe^{B1} is not deleted, then 2-4 Zn^{2+} ions are bound to each hexamer of the insulin derivative.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; the A21 and the B3

amino acid residues are, independently, any amino acid residues which can be coded for by the genetic code except Lys, Arg and Cys, with the proviso that if the B30 amino acid residue is Ala or Thr, then at least one of the residues A21 and B3 is different from Asn; Phe^{B1} may be deleted; and the ϵ-amino group of Lys^{B29} has a lipophilic substituent which comprises at least 6 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; the A21 and the B3 amino acid residues are, independently, any amino acid residues which can be coded for by the genetic code except Lys, Arg and Cys; Phe^{B1} may be deleted; the ϵ-amino group of Lys^{B29} has a lipophilic substituent which comprises at least 6 carbon atoms; and 2-4 Zn^{2+} ions are bound to each insulin hexamer.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is Asp.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is Glu.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is Thr.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is a lipophilic amino acid having at least 10 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is a lipophilic α-amino acid having from 10 to 24 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is a straight chain, saturated, aliphatic α-amino acid having from 10 to 24 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is D- or L-N^{ε}-dodecanoyllysine.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α-amino decanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α-amino undecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α-amino dodecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α-amino tridecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α-amino tetradecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α-amino pentadecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α-amino hexadecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is an α-amino acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the A21 amino acid residue is Ala.

In another preferred embodiment, the invention relates to a human insulin derivative in which the A21 amino acid residue is Gln.

In another preferred embodiment, the invention relates to a human insulin derivative in which the A21 amino acid residue is Gly.

In another preferred embodiment, the invention relates to a human insulin derivative in which the A21 amino acid residue is Ser.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B3 amino acid residue is Asp.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B3 amino acid residue is Gln.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B3 amino acid residue is Thr.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ-amino group of Lys^{B29} has a lipophilic substituent which is an acyl group corresponding to a carboxylic acid having at least 6 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ-amino group of Lys^{B29} has a lipophilic substituent which is an acyl group, branched or unbranched, which corresponds to a carboxylic acid having a chain of carbon atoms 8 to 24 atoms long.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ-amino group of Lys^{B29} has a lipophilic substituent which is an acyl group corresponding to a fatty acid having at least 6 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ-amino group of Lys^{B29} has a lipophilic substituent which is an acyl group corresponding to a linear, saturated carboxylic acid having from 6 to 24 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ-amino group of Lys^{B29} has a lipophilic substituent which is an acyl group corresponding to a linear, saturated carboxylic acid having from 8 to 12 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ-amino group of Lys^{B29} has a lipophilic substituent which is an acyl group corresponding to a linear, saturated carboxylic acid having from 10 to 16 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ-amino group of Lys^{B29} has

a lipophilic substituent which is an oligo oxyethylene group comprising up to 10, preferably up to 5, oxyethylene units.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ-amino group of Lys^{B29} has a lipophilic substituent which is an oligo oxypropylene group comprising up to 10, preferably up to 5, oxypropylene units.

In another preferred embodiment, the invention relates to a human insulin derivative in which each insulin hexamer binds 2 Zn^{2+} ions.

In another preferred embodiment, the invention relates to a human insulin derivative in which each insulin hexamer binds 3 Zn^{2+} ions.

In another preferred embodiment, the invention relates to a human insulin derivative in which each insulin hexamer binds 4 Zn^{2+} ions.

In another preferred embodiment, the invention relates to the use of a human insulin derivative according to the invention for the preparation of a medicament for treating diabetes.

In another preferred embodiment, the invention relates to a pharmaceutical composition for the treatment of diabetes in a patient in need of such a treatment comprising a therapeutically effective amount of a human insulin derivative according to the invention together with a pharmaceutically acceptable carrier.

In another preferred embodiment, the invention relates to a pharmaceutical composition for the treatment of diabetes in a patient in need of such a treatment comprising a therapeutically effective amount of a human insulin derivative according to the invention, in mixture with an insulin or an

insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

In another preferred embodiment, the invention relates to a pharmaceutical composition comprising a human insulin derivative according to the invention which is soluble at physiological pH values.

In another preferred embodiment, the invention relates to a pharmaceutical composition comprising a human insulin derivative according to the invention which is soluble at pH values in the interval from about 6.5 to about 8.5.

In another preferred embodiment, the invention relates to a protracted pharmaceutical composition comprising a human insulin derivative according to the invention.

In another preferred embodiment, the invention relates to a pharmaceutical composition which is a solution containing from about 120 nmol/ml to about 1200 nmol/ml, preferably about 600 nmol/ml of a human insulin derivative according to the invention.

In another preferred embodiment, the invention relates to a method of treating diabetes in a patient in need of such a treatment comprising administering to the patient a therapeutically effective amount of an insulin derivative according to this invention together with a pharmaceutically acceptable carrier.

In another preferred embodiment, the invention relates to a method of treating diabetes in a patient in need of such a treatment comprising administering to the patient a therapeutically effective amount of an insulin derivative according to this invention, in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

Examples of preferred human insulin derivatives according to the present invention in which no Zn^{2+} ions are bound are the following:

$N^{\epsilon B29}$-tridecanoyl des(B30) human insulin,
$N^{\epsilon B29}$-tetradecanoyl des(B30) human insulin,
$N^{\epsilon B29}$-decanoyl des(B30) human insulin,
$N^{\epsilon B29}$-dodecanoyl des(B30) human insulin,
$N^{\epsilon B29}$-tridecanoyl Gly^{A21} des(B30) human insulin,
$N^{\epsilon B29}$-tetradecanoyl Gly^{A21} des(B30) human insulin,
$N^{\epsilon B29}$-decanoyl Gly^{A21} des(B30) human insulin,
$N^{\epsilon B29}$-dodecanoyl Gly^{A21} des(B30) human insulin,
$N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-tridecanoyl Ala^{A21} des(B30) human insulin,
$N^{\epsilon B29}$-tetradecanoyl Ala^{A21} des(B30) human insulin,
$N^{\epsilon B29}$-decanoyl Ala^{A21} des(B30) human insulin,
$N^{\epsilon B29}$-dodecanoyl Ala^{A21} des(B30) human insulin,
$N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-tridecanoyl Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-tetradecanoyl Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-decanoyl Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-dodecanoyl Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-tridecanoyl Gly^{A21} human insulin,
$N^{\epsilon B29}$-tetradecanoyl Gly^{A21} human insulin,
$N^{\epsilon B29}$-decanoyl Gly^{A21} human insulin,
$N^{\epsilon B29}$-dodecanoyl Gly^{A21} human insulin,
$N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} human insulin,
$N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} human insulin,
$N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} human insulin,
$N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} human insulin,
$N^{\epsilon B29}$-tridecanoyl Ala^{A21} human insulin,

$N^{\epsilon B29}$-tetradecanoyl Ala^{A21} human insulin,
$N^{\epsilon B29}$-decanoyl Ala^{A21} human insulin,
$N^{\epsilon B29}$-dodecanoyl Ala^{A21} human insulin,
$N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} human insulin,
$N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} human insulin,
$N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} human insulin,
$N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} human insulin,
$N^{\epsilon B29}$-tridecanoyl Gln^{B3} human insulin,
$N^{\epsilon B29}$-tetradecanoyl Gln^{B3} human insulin,
$N^{\epsilon B29}$-decanoyl Gln^{B3} human insulin,
$N^{\epsilon B29}$-dodecanoyl Gln^{B3} human insulin,
$N^{\epsilon B29}$-tridecanoyl Glu^{B30} human insulin,
$N^{\epsilon B29}$-tetradecanoyl Glu^{B30} human insulin,
$N^{\epsilon B29}$-decanoyl Glu^{B30} human insulin,
$N^{\epsilon B29}$-dodecanoyl Glu^{B30} human insulin,
$N^{\epsilon B29}$-tridecanoyl Gly^{A21} Glu^{B30} human insulin,
$N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Glu^{B30} human insulin,
$N^{\epsilon B29}$-decanoyl Gly^{A21} Glu^{B30} human insulin,
$N^{\epsilon B29}$-dodecanoyl Gly^{A21} Glu^{B30} human insulin,
$N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin,
$N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin,
$N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin,
$N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin,
$N^{\epsilon B29}$-tridecanoyl Ala^{A21} Glu^{B30} human insulin,
$N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Glu^{B30} human insulin,
$N^{\epsilon B29}$-decanoyl Ala^{A21} Glu^{B30} human insulin,
$N^{\epsilon B29}$-dodecanoyl Ala^{A21} Glu^{B30} human insulin,
$N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin,
$N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin,
$N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin,
$N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin,
$N^{\epsilon B29}$-tridecanoyl Gln^{B3} Glu^{B30} human insulin,
$N^{\epsilon B29}$-tetradecanoyl Gln^{B3} Glu^{B30} human insulin,
$N^{\epsilon B29}$-decanoyl Gln^{B3} Glu^{B30} human insulin and
$N^{\epsilon B29}$-dodecanoyl Gln^{B3} Glu^{B30} human insulin.

Examples of preferred human insulin derivatives according to the present invention in which two Zn^{2+} ions are bound per insulin hexamer are the following:

($N^{\epsilon B29}$-tridecanoyl des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,

($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gln^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,

($N^{\epsilon B29}$-decanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$ and
($N^{\epsilon B29}$-dodecanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$.

Examples of preferred human insulin derivatives according to the present invention in which three Zn^{2+} ions are bound per insulin hexamer are the following:

($N^{\epsilon B29}$-tridecanoyl des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} human insulin)$_6$, $3Zn^{2+}$,

($N^{\epsilon B29}$-decanoyl Gly^{A21} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,

($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$ and
($N^{\epsilon B29}$-dodecanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$.

Examples of preferred human insulin derivatives according to the present invention in which four Zn^{2+} ions are bound per insulin hexamer are the following:

($N^{\epsilon B29}$-tridecanoyl des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl human insulin)$_6$, $4Zn^{2+}$,

($N^{\epsilon B29}$-dodecanoyl human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,

($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$ and
($N^{\epsilon B29}$-dodecanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is further illustrated with reference to the appended drawings wherein

Fig. 1 shows the construction of the plasmid pEA5.3.2;

Fig. 2 shows the construction of the plasmid pEA108; and

Fig. 3 shows the construction of the plasmid pEA113.

DETAILED DESCRIPTION OF THE INVENTION

Terminology

The three letter codes and one letter codes for the amino acid residues used herein are those stated in J. Biol. Chem. 243, p. 3558 (1968).

In the DNA sequences, A is adenine, C is cytosine, G is guanine, and T is thymine.

The following acronyms are used:

DMSO for dimethyl sulphoxide, DMF for dimethylformamide, Boc for tert-butoxycarbonyl, RP-HPLC for reversed phase high performance liquid chromatography, X-OSu is an N-hydroxysuccinimid ester, X is an acyl group, and TFA for trifluoroacetic acid.

Preparation of lipophilic insulin derivatives

The insulin derivatives according to the present invention can be prepared i.a. as described in the following:

1. Insulin derivatives featuring in position B30 an amino acid residue which can be coded for by the genetic code, e.g. threonine (human insulin) or alanine (porcine insulin).

1.1 Starting from human insulin.

Human insulin is treated with a Boc-reagent (e.g. di-tert-butyl dicarbonate) to form (A1,B1)-diBoc human insulin, i.e., human insulin in which the N-terminal end of both chains are protected by a Boc-group. After an optional purification, e.g. by HPLC, an acyl group is introduced in the ϵ-amino group of Lys^{B29} by allowing the product to react with a N-hydroxysuccinimide ester of the formula X-OSu wherein X is the acyl group to be introduced. In the final step, TFA is used to remove the Boc-groups and the product, $N^{\epsilon B29}$-X human insulin, is isolated.

1.2 Starting from a single chain insulin precursor.

A single chain insulin precursor, extended in position B1 with an extension (Ext) which is connected to B1 via an arginine residue and in which the bridge from B30 to A1 is an arginine residue, i.e. a compound of the general formula Ext-Arg-B(1-30)-Arg-A(1-21), can be used as starting material. Acylation of this starting material with a N-hydroxysuccinimide ester of the general formula X-OSu wherein X is an acyl group, introduces the acyl group X in the ϵ-amino group of Lys^{B29} and in the N-terminal amino group of the precursor. On treating this acylated precursor of the formula ($N^{\epsilon B29}$-X),X-Ext-Arg-B(1-30)-

Arg-A(1-21) with trypsin in a mixture of water and a suitable water-miscible organic solvent, e.g. DMF, DMSO or a lower alcohol, an intermediate of the formula ($N^{\epsilon B29}$-X),Arg^{B31} insulin is obtained. Treating this intermediate with carboxypeptidase B yields the desired product, ($N^{\epsilon B29}$-X) insulin.

2. Insulin derivatives with no amino acid residue in position B30, i.e. des(B30) insulins.

2.1 Starting from human insulin or porcine insulin.

On treatment with carboxypeptidase A in ammonium buffer, human insulin and porcine insulin both yield des(B30) insulin. After an optional purification, the des(B30) insulin is treated with a Boc-reagent (e.g. di-tert-butyl dicarbonate) to form (A1,B1)-diBoc des(B30) insulin, i.e., des(B30) insulin in which the N-terminal end of both chains are protected by a Boc-group. After an optional purification, e.g. by HPLC, an acyl group is introduced in the ϵ-amino group of Lys^{B29} by allowing the product to react with a N-hydroxysuccinimide ester of the formula X-OSu wherein X is the acyl group to be introduced. In the final step, TFA is used to remove the Boc-groups and the product, ($N^{\epsilon B29}$-X) des(B30) insulin, is isolated.

2.2 Starting from a single chain human insulin precursor.

A single chain human insulin precursor, which is extended in position B1 with an extension (Ext) which is connected to B1 via an arginine residue and which has a bridge from B30 to A1 can be a useful starting material. Preferably, the bridge is a peptide of the formula Y_n-Arg, where Y is a codable amino acid except lysine and arginine, and n is zero or an integer between 1 and 35. When n>1, the Y's may designate different amino acids. Preferred examples of the bridge from B30 to A1 are: AlaAlaArg, SerArg, SerAspAspAlaArg and Arg (European Patent No.

163529). Treatment of such a precursor of the general formula Ext-Arg-B(1-30)-Y_n-Arg-A(1-21) with a lysyl endopeptidase, e.g. Achromobacter lyticus protease, yields Ext-Arg-B(1-29) Thr-Y_n-Arg-A(1-21) des(B30) insulin. Acylation of this intermediate with a N-hydroxysuccinimide ester of the general formula X-OSu wherein X is an acyl group, introduces the acyl group X in the ϵ-amino group of Lys^{B29}, and in the N-terminal amino group of the A-chain and the B-chain to give ($N^{\epsilon B29}$-X) X-Ext-Arg-B(1-29) X-Thr-Y_n-Arg-A(1-21) des(B30) insulin. This intermediate on treatment with trypsin in mixture of water and a suitable organic solvent, e.g. DMF, DMSO or a lower alcohol, gives the desired derivative, ($N^{\epsilon B29}$-X) des(B30) human insulin.

Data on $N^{\epsilon B29}$ modified insulins.

Certain experimental data on $N^{\epsilon B29}$ modified insulins are given in Table 1.

The lipophilicity of an insulin derivative relative to human insulin, k'_{rel}, was measured on a LiChrosorb RP18 (5μm, 250x4 mm) HPLC column by isocratic elution at 40°C using mixtures of A) 0.1 M sodium phosphate buffer, pH 7.3, containing 10% acetonitrile, and B) 50% acetonitrile in water as eluents. The elution was monitored by following the UV absorption of the eluate at 214 nm. Void time, t_0, was found by injecting 0.1 mM sodium nitrate. Retention time for human insulin, t_{human}, was adjusted to at least $2t_0$ by varying the ratio between the A and B solutions. $k'_{rel} = (t_{derivative}-t_0)/(t_{human}-t_0)$.

The degree of prolongation of the blood glucose lowering effect was studied in rabbits. Each insulin derivative was tested by subcutaneous injection of 12 nmol thereof in each of six rabbits in the single day retardation test. Blood sampling for glucose analysis was performed before injection and at 1, 2, 4 and 6 hours after injection. The glucose values found are expressed as percent of initial values. The Index of

Protraction, which was calculated from the blood glucose values, is the scaled Index of Protraction (prolongation), see p. 211 in Markussen et al., Protein Engineering 1 (1987) 205-213. The formula has been scaled to render a value of 100 with bovine ultralente insulin and a value of 0 with Actrapid® insulin (Novo Nordisk A/S, 2880 Bagsvaerd, Denmark).

The insulin derivatives listed in Table 1 were administered in solutions containing 3 Zn^{2+} per insulin hexamer, except those specifically indicated to be Zn-free.

For the very protracted analogues the rabbit model is inadequate because the decrease in blood glucose from initial is too small to estimate the index of protraction. The prolongation of such analogues is better characterized by the disappearance rate in pigs. $T_{50\%}$ is the time when 50% of the A14 Tyr(^{125}I) analogue has disappeared from the site of injection as measured with an external γ-counter (Ribel, U et al., The Pig as a Model for Subcutaneous Absorption in Man. In: M. serrano-Rios and P.J. Lefebre (Eds): Diabetes 1985; Proceedings of the 12th Congress of the International Diabetes Federation, Madrid, Spain, 1985 (Excerpta Medica, Amsterdam, (1986) 891-96).

In Table 2 are given the $T_{50\%}$ values of a series of very protracted insulin analogues. The analogues were administered in solutions containing 3 Zn^{2+} per insulin hexamer.

Table 1

Insulin Derivative *)	Relative Lipophilicity	Blood glucose, % of initial				Index of protraction
		1h	2h	4h	6h	
$N^{\epsilon B29}$-benzoyl insulin	1.14					
$N^{\epsilon B29}$-phenylacetyl insulin (Zn-free)	1.28	55.4	58.9	88.8	90.1	10
$N^{\epsilon B29}$-cyclohexylacetyl insulin	1.90	53.1	49.6	66.9	81.1	28
$N^{\epsilon B29}$-cyclohexylpropionyl insulin	3.29	55.5	47.6	61.5	73.0	39
$N^{\epsilon B29}$-cyclohexylvaleroyl insulin	9.87	65.0	58.3	65.7	71.0	49
$N^{\epsilon B29}$-octanoyl insulin	3.97	57.1	54.8	69.0	78.9	33
$N^{\epsilon B29}$-decanoyl, des(B30) insulin	11.0	74.3	65.0	60.9	64.1	65
$N^{\epsilon B29}$-decanoyl insulin	12.3	73.3	59.4	64.9	68.0	60
$N^{\epsilon B29}$-undecanoyl, des(B30) insulin	19.7	88.1	80.0	72.1	72.1	80
$N^{\epsilon B29}$-lauroyl, des(B30) insulin	37.0	91.4	90.0	84.2	83.9	78
$N^{\epsilon B29}$-myristoyl insulin	113	98.5	92.0	83.9	84.5	97
$N^{\epsilon B29}$-choloyl insulin	7.64	58.2	53.2	69.0	88.5	20
$N^{\epsilon B29}$-7-deoxycholoyl insulin (Zn-free)	24.4	76.5	65.2	77.4	87.4	35
$N^{\epsilon B29}$-lithocholoyl insulin (Zn-free)	51.6	98.3	92.3	100.5	93.4	115
$N^{\epsilon B29}$-4-benzoyl-phenylalanyl insulin	2.51	53.9	58.7	74.4	89.0	14
$N^{\epsilon B29}$-3,5-diiodotyrosyl insulin	1.07	53.9	48.3	60.8	82.1	27
$N^{\epsilon B29}$-L-thyroxyl insulin	8.00					

Table 2

Derivative of Human Insulin	Relative hydrophobicity	Subcutaneous disappearance in pigs
600 μM, $3Zn^{2+}$/hexamer, phenol 0.3%, glycerol 1.6%, pH 7.5	k'_{rel}	$T_{50\%}$, hours
$N^{\epsilon B29}$decanoyl des(B30) insulin	11.0	5.6
$N^{\epsilon B29}$undecanoyl des(B30) insulin	19.7	6.9
$N^{\epsilon B29}$lauroyl des(B30) insulin	37	10.1
$N^{\epsilon B29}$tridecanoyl des(B30) insulin	65	12.9
$N^{\epsilon B29}$myristoyl des(B30) insulin	113	13.8
$N^{\epsilon B29}$palmitoyl des(B30) insulin	346	12.4
$N^{\epsilon B29}$succinimido-myristic acid insulin	10.5	13.6
$N^{\epsilon B29}$myristoyl insulin	113	11.9
Human NPH		10

Solubility

The solubility of all the $N^{\epsilon B29}$ modified insulins mentioned in Table 1, which contain 3 Zn^{2+} ions per insulin hexamer, exceeds 600 nmol/ml in a neutral (pH 7.5), aqueous, pharmaceutical formulation which further comprises 0.3% phenol as preservative, and 1.6% glycerol to achieve isotonicity. 600 nmol/ml is the concentration of human insulin found in the 100 IU/ml compositions usually employed in the clinic.

The ε-B29 amino group can be a component of an amide bond, a sulphonamide bond, a carbamide, a thiocarbamide, or a carbamate. The lipophilic substituent carried by the ε-B29 amino group can also be an alkyl group.

Pharmaceutical compositions containing a human insulin derivative according to the present invention may be administered parenterally to patients in need of such a treatment. Parenteral administration may be performed by subcutaneous, intramuscular or intravenous injection by means of a syringe, optionally a pen-like syringe. Alternatively, parenteral administration can be performed by means of an infusion pump. A further option is a composition which may be a powder or a liquid for the administration of the human insulin derivative in the form of a nasal spray.

The injectable human insulin compositions of the invention can be prepared using the conventional techniques of the pharmaceutical industry which involves dissolving and mixing the ingredients as appropriate to give the desired end product.

Thus, according to one procedure, the human insulin derivative is dissolved in an amount of water which is somewhat less than the final volume of the composition to be prepared. An isotonic agent, a preservative and a buffer is added as required and the pH value of the solution is adjusted - if necessary - using an acid, e.g. hydrochloric acid, or a base, e.g. aqueous sodium hydroxide as needed. Finally, the volume of the solution is adjusted with water to give the desired concentration of the ingredients.

Examples of isotonic agents are sodium chloride, mannitol and glycerol.

Examples of preservatives are phenol, m-cresol, methyl p-hydroxybenzoate and benzyl alcohol.

Examples of suitable buffers are sodium acetate and sodium phosphate.

A composition for nasal administration of an insulin derivative according to the present invention may, for example, be prepared as described in European Patent No. 272097 (to Novo Nordisk A/S).

The insulin compositions of this invention can be used in the treatment of diabetes. The optimal dose level for any patient will depend on a variety of factors including the efficacy of the specific human insulin derivative employed, the age, body weight, physical activity, and diet of the patient, on a possible combination with other drugs, and on the severity of the case of diabetes. It is recommended that the daily dosage of the human insulin derivative of this invention be determined for each individual patient by those skilled in the art in a similar way as for known insulin compositions.

Where expedient, the human insulin derivatives of this invention may be used in mixture with other types of insulin, e.g. human insulin or porcine insulin or insulin analogues with a more rapid onset of action. Examples of such insulin analogues are described e.g. in the European patent applications having the publication Nos. EP 214826 (Novo Nordisk A/S), EP 375437 (Novo Nordisk A/S) and EP 383472 (Eli Lilly & Co.).

The present invention is further illustrated by the following examples which, however, are not to be construed as limiting the scope of protection. The features disclosed in the foregoing description and in the following examples may, both separately and in any combination thereof, be material for realizing the invention in diverse forms thereof.

EXAMPLES

Plasmids and DNA material

All expression plasmids are of the cPOT type. Such plasmids are described in EP patent application No. 171 142 and are characterized in containing the Schizosaccharomyces pombe triose phosphate isomerase gene (POT) for the purpose of plasmid selection and stabilization. A plasmid containing the POT-gene is available from a deposited E. coli strain (ATCC 39685). The plasmids furthermore contain the S. cerevisiae triose phosphate isomerase promoter and terminator (P_{TPI} and T_{TPI}). They are identical to pMT742 (Egel-Mitani, M. et al., Gene 73 (1988) 113-120) (see Fig. 1) except for the region defined by the ECoRI-XbaI restriction sites encompassing the coding region for signal/leader/product.

Synthetic DNA fragments were synthesized on an automatic DNA synthesizer (Applied Biosystems model 380A) using phosphoramidite chemistry and commercially available reagents (Beaucage, S.L. and Caruthers, M.H., Tetrahedron Letters 22 (1981) 1859-1869).

All other methods and materials used are common state of the art knowledge (see, e.g. Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 1989).

Analytical

Molecular masses of the insulins prepared were obtained by MS (mass spectroscopy), either by PDMS (plasma desorption mass spectrometry) using a Bio-Ion 20 instrument (Bio-Ion Nordic AB, Uppsala, Sweden) or by ESMS (electrospray mass spectrometry) using an API III Biomolecular Mass Analyzer (Perkin-Elmer Sciex Instruments, Thornhill, Canada).

EXAMPLE 1

Synthesis of Ala^{A21} Asp^{B3} human insulin precursor from Yeast strain yEA002 using the LaC212spx3 signal/leader.

The following oligonucleotides were synthesized:

#98 5'-TGGCTAAGAGATTCGTTGACCAACACTTGTGCGGTTCTCA
CTTGGTTGAAGCTTTGTACTTGGTTTGTGGTGAA
AGAGGTTTCTTCTACACTCCAAAGTCTGACGACGCT-3' (Asp^{B3})
(SEQ ID NO:3)

#128 5'-CTGCGGGCTGCGTCTAAGCACAGTAGTTTTCCAATTGGTACAA
AGAACAGATAGAAGTACAACATTGTTCAACGATACCCTTAGCGTC
GTCAGACTTTGG-3' (Ala^{A21}) (SEQ ID NO:4)

#126 5'-GTCGCCATGGCTAAGAGATTCGTTG-3' (Asp^{B3})
(SEQ ID NO:5)

#16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3' (SEQ ID NO:6)

The following Polymerase Chain Reaction (PCR) was performed using the Gene Amp PCR reagent kit (Perkin Elmer, 761 Main Avewalk, CT 06859, USA) according to the manufacturer's instructions. In all cases, the PCR mixture was overlayed with 100 µl of mineral oil (Sigma Chemical Co., St. Louis, MO, USA).

2.5 µl of oligonucleotide #98 (2.5 pmol)
2.5 µl of oligonucleotide #128 (2.5 pmol)
10 µl of 10X PCR buffer
16 µl of dNTP mix
0.5 µl of Taq enzyme
58.5 µl of water

One cycle was performed: 94°C for 45 sec., 49°C for 1 min, 72°C for 2 min.

Subsequently, 5µl of oligonucleotides #16 and #126 was added and 15 cycles were performed: 94°C for 45 sec., 45°C for 1 min, 72°C for 1.5 min. The PCR mixture was loaded onto a 2.5 %

agarose gel and subjected to electrophoresis using standard techniques (Sambrook et al., Molecular cloning, Cold Spring Harbour Laboratory Press, 1989). The resulting DNA fragment was cut out of the agarose gel and isolated using the Gene Clean Kit (Bio 101 Inc., PO BOX 2284, La Jolla, CA 92038, USA) according to the manufacturer's instructions. The purified PCR DNA fragment was dissolved in 10 µl of water and restriction endonuclease buffer and cut with the restriction endonucleases NcoI and Xba I according to standard techniques, run on a 2.5% agarose gel and purified using the Gene Clean Kit as described.

The plasmid pAK188 consists of a DNA sequence of 412 bp composed of a EcoRI/NcoI fragment encoding the synthetic yeast signal/leader gene LaC212spx3 (described in Example 3 of WO 89/02463) followed by a synthetic NcoI/XbaI fragment encoding the insulin precursor MI5, which has a SerAspAspAlaLys bridge connecting the B29 and the A1 amino acid residues (see SEQ ID NOS. 14, 15 and 16), inserted into the EcoRI/XbaI fragment of the vector (phagemid) pBLUESCRIPT IIsk(+/-) (Stratagene, USA). The plasmid pAK188 is shown in Fig. 1.

The plasmid pAK188 was also cut with the restriction endonucleases NcoI and XbaI and the vector fragment of 3139 bp isolated. The two DNA fragments were ligated together using T4 DNA ligase and standard conditions (Sambrook et al., Molecular Cloning, Cold Spring Harbour Laboratory Press, 1989). The ligation mixture was transformed into a competent E. coli strain (R-, M+) followed by selection for ampicillin resistance. Plasmids were isolated from the resulting E. coli colonies using standard DNA miniprep technique (Sambrook et al., Molecular Cloning, Cold Spring Harbour Laboratory Press, 1989), checked with appropriate restrictions endonucleases i.e. EcoRI, Xba I, NcoI and HpaI. The selected plasmid was shown by DNA sequencing analyses (Sequenase, U.S. Biochemical Corp.) to contain the correct sequence for the Ala^{A21}, Asp^{B3} human insulin precursor and named pEA5.3.

The plasmid pKFN1627 is an E. coli - S. cerevisiae shuttle vector, identical to plasmid pKFN1003 described in EP patent No. 375718, except for a short DNA sequence upstream from the unique XbaI site. In pKFN1003, this sequence is a 178 bp fragment encoding a synthetic aprotinin gene fused in-frame to the yeast mating factor alpha 1 signal-leader sequence. In pKFN1627, the corresponding 184 bp sequence encodes the insulin precursor MI5 (Glu^{B1}, Glu^{B28}) (i.e. B(1-29, Glu^{B1},Glu^{B28})-SerAspAspAlaLys-A(1-21) fused in-frame to the mating factor alpha 1 sequence (see SEQ ID NOS. 17, 18 and 19). The vector pKFN1627 is shown in Fig. 1.

pEA5.3 was cut with the restriction endonucleases EcoRI and XbaI and the resulting DNA fragment of 412 bp was isolated. The yeast expression vector pKFN1627 was cut with the restriction endonucleases NcoI and XbaI and with NcoI and EcoRI and the DNA fragment of 9273 bp was isolated from the first digestion and the DNA fragment of 1644 bp was isolated from the second. The 412 bp EcoRI/XbaI fragment was then ligated to the two other fragments, that is the 9273 bp NcoI I/XbaI fragment and the 1644 bp NcoI/EcoRI fragment using standard techniques.

The ligation mixture was transformed into E. coli as described above. Plasmid from the resulting E. coli was isolated using standard techniques, and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI, Hpa I. The selected plasmid was shown by DNA sequence analysis (using the Sequenase kit as described by the manufacturer, U.S. Biochemical) to contain the correct sequence for the Ala^{A21} Asp^{B3} human insulin precursor DNA and to be inserted after the DNA encoding the LaC212spx3 signal/leader DNA. The plasmid was named pEA5.3.2 and is shown in Fig. 1. The DNA sequence encoding the LaC212spx3 signal/leader/Ala^{A21} Asp^{B3} human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 20, 21 and 22. The plasmid pEA5.3.2 was transformed into S. cerevisiae strain MT663 as described in European patent

application having the publication No. 214826 and the resulting strain was named yEA002.

EXAMPLE 2

Synthesis of Ala^{A21} Thr^{B3} human insulin precursor from Yeast strain yEA005 using the LaC212spx3 signal/leader.

The following oligonucleotides were synthesized:

#101 5'-TGGCTAAGAGATTCGTTACTCAACACTTGTGCGGTTCTCACTT GGTTGAAGCTTTGTACTTGGTTTGTGGTGAAAGAGGTTTCTTCTACA CTCCAAAGTCTGACGACGCT-3' (Thr^{B3}) (SEQ ID NO:7)

#128 5'-CTGCGGGCTGCGTCTAAGCACAGTAGTTTTCCAATTGGTACAAA GAACAGATAGAAGTACAACATTGTTCAACGATACCCTTAGCGTCG TCAGACTTTGG-3' (Ala^{A21}) (SEQ ID NO:4)

#15 5'-GTCGCCATGGCTAAGAGATTCGTTA-3' (Thr^{B3}) (SEQ ID NO:8)

#16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3' (SEQ ID NO:6)

The DNA encoding Ala^{A21} Thr^{B3} human insulin precursor was constructed in the same manner as described for the DNA encoding Ala^{A21} Asp^{B3} human insulin precursor in Example 1. The DNA sequence encoding the LaC212spx3 signal/leader/Ala^{A21} Thr^{B3} human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 23, 24 and 25. The plasmid pEA8.1.1 was shown to contain the desired sequence, transformed into S. cerevisiae strain MT663 as described in Example 1 and the resulting strain was named yEA005.

EXAMPLE 3

Synthesis of Gly^{A21} Asp^{B3} human insulin precursor from Yeast strain yEA007 using the LaC212spx3 signal/leader.

The following oligonucleotides were synthesized:

#98 5'-TGGCTAAGAGATTCGTTGACCAACACTTGTGCGGTTCTCACTTG
GTTGAAGCTTTGTACTTGGTTTGTGGTGAAAGAGGTTTCTTCT
ACACTCCAAAGTCTGACGACGCT-3' (Asp^{B3}) (SEQ ID NO:3)

#127 5'-CTGCGGGCTGCGTCTAACCACAGTAGTTTTCCAATTGGTACAA
AGAACAGATAGAAGTACAACATTGTTCAACGATACCCT
TAGCGTCGTCAGACTTTGG-3' (Gly^{A21}) (SEQ ID NO:9)

#126 5'-GTCGCCATGGCTAAGAGATTCGTTG-3' (Asp^{B3}) (SEQ ID NO:5)

#16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3' (SEQ ID NO:6)

The DNA encoding Gly^{A21} Asp^{B3} human insulin precursor was constructed in the same manner as described for the DNA encoding Ala^{A21} Asp^{B3} human insulin precursor in Example 1. The DNA sequence encoding the LaC212spx3 signal/leader/Gly^{A21} Asp^{B3} human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 26, 27 and 28. The plasmid pEA1.5.6 was shown to contain the desired sequence, transformed into S. cerevisiae strain MT663 as described in Example 1 and the resulting strain was named yEA007.

EXAMPLE 4

Synthesis of Gly^{A21} Thr^{B3} human insulin precursor from Yeast strain yEA006 using the LaC212spx3 signal/leader.

The following oligonucleotides were synthesized:

#101 5'-TGGCTAAGAGATTCGTTACTCAACACTTGTGCGGTTCTCACTT
GGTTGAAGCTTTGTACTTGGTTTGTGGTGAAAGAGGTTTCTTCTACA
CTCCAAAGTCTGACGACGCT-3' (Thr^{B3}) (SEQ ID NO:7)

#127 5'-CTGCGGGCTGCGTCTAACCACAGTAGTTTTCCAATTGGTACAA
AGAACAGATAGAAGTACAACATTGTTCAACGATACCCT
TAGCGTCGTCAGACTTTGG-3' (Gly^{A21}) (SEQ ID NO:9)

#15 5'-GTCGCCATGGCTAAGAGATTCGTTA-3' (Thr^{B3}) (SEQ ID NO:8)

#16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3' (SEQ ID NO:6)

The DNA encoding GlyA21 Thr^{B3} human insulin precursor was constructed in the same manner as described for the DNA encoding Ala^{A21} Asp^{B3} human insulin precursor in Example 1. The DNA sequence encoding the LaC212spx3 signal/leader/Gly^{A21} Thr^{B3} human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 29, 30 and 31. The plasmid pEA4.4.11 was shown to contain the desired DNA sequence, transformed into S. cerevisiae strain MT663 as described in Example 1 and the resulting strain was named yEA006.

EXAMPLE 5

Synthesis of Arg^{B-1} Arg^{B31} single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaArg) from Yeast strain yEA113 using the alpha factor leader.

A)

The following oligonucleotides were synthesized:

#220 5'-ACGTACGTTCTAGAGCCTGCGGGCTGC-3' (SEQ ID NO:10)

#263 5'-CACTTGGTTGAAGCTTTGTACTTGGTTTGTGGTGAAAGAGGTTTC TTCTACACTCCAAAGACTAGAGGTATCGTTGAA-3' (SEQ ID NO:11)

#307 5'-GCTAACGTCGCCATGGCTAAGAGAGAAGAAGCTGAAGCTGAAGCT AGATTCGTTAACCAACAC-3' (SEQ ID NO:12)

The following Polymerase Chain Reaction (PCR) was performed using the Gene Amp PCR reagent kit (Perkin Elmer, 761 Main Avewalk, CT 06859, USA) according to the manufacturer's instructions. In all cases, the PCR mixture was overlayed with 100 µl of mineral oil (Sigma Chemical Co, St. Louis, MO, USA). The plasmid pAK220 (which is identical to pAK188) consists of a DNA sequence of 412 bp encoding the synthetic yeast signal/leader LaC212spx3 (described in Example 3 of WO 89/02463) followed by the insulin precursor MI5 (see SEQ ID NOS. 14, 15 and 16) inserted into the vector (phagemid) pBLUESCRIPT IIsk(+/-) (Stratagene, USA).

5 µl of oligonucleotide #220 (100 pmol)
5 µl of oligonucleotide #263 (100 pmol)
10 µl of 10X PCR buffer
16 µl of dNTP mix
0.5 µl of Taq enzyme
0.5 µl of pAK220 plasmid (identical to pAK188) as template (0.2 µg of DNA)
63 µl of water

A total of 16 cycles were performed, each cycle comprising 1 minute at 95°C; 1 minute at 40°C; and 2 minutes at 72°C. The PCR mixture was then loaded onto a 2% agarose gel and subjected to electrophoresis using standard techniques. The resulting DNA fragment was cut out of the agarose gel and isolated using the Gene Clean kit (Bio 101 Inc., PO BOX 2284, La Jolla, CA 92038, USA) according to the manufacture's instructions. The purified PCR DNA fragment was dissolved in 10 µl of water and restriction endonuclease buffer and cut with the restriction endonucleases HindIII and XbaI according to standard techniques. The HindIII/XbaI DNA fragment was purified using The Gene Clean Kit as described.

The plasmid pAK406 consists of a DNA sequence of 520 bp comprising an EcoRI/HindIII fragment derived from pMT636 (described in WO 90/10075) encoding the yeast alpha factor leader and part of the insulin precursor ligated to the HindIII/XbaI fragment from pAK188 encoding the rest of the insulin precursor MI5 (see SEQ ID NOS. 32, 33 and 34) inserted into the vector cPOT. The vector pAK406 is shown in Fig. 2.

The plasmid pAK233 consists of a DNA sequence of 412 bp encoding the synthetic yeast signal/leader LaC212spx3 (described in Example 3 of WO 89/02463) followed by the gene for the insulin precursor B(1-29)-GluLysArg-A(1-21) (A21-Gly) (see SEQ ID NOS. 35, 36 and 37) inserted into the vector cPOT. The plasmid pAK233 is shown in Fig. 2.

The plasmid pAK233 was cut with the restriction endonucleases NcoI and XbaI and the vector fragment of 9273 bp isolated. The plasmid pAK406 was cut with the restriction endonucleases NcoI and HindIII and the vector fragment of 2012 bp isolated. These two DNA fragments were ligated together with the HindIII/XbaI PCR fragment using T4 DNA ligase and standard conditions. The ligation mixture was then transformed into a competent E. coli strain (R-, M+) followed by selection for ampicillin resistance. Plasmids were isolated from the resulting E. coli colonies using a standard DNA miniprep technique and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI, HindIII. The selected plasmid was shown by DNA sequencing analyses to contain the correct sequence for the Arg^{B31} single chain human insulin precursor DNA and to be inserted after the DNA encoding the S. cerevisiae alpha factor DNA. The plasmid was named pEA108 and is shown in Fig. 2. The DNA sequence encoding the alpha factor leader/Arg^{B31} single chain human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 38, 39 and 40. The plasmid pEA 108 was transformed into S. cerevisiae strain MT663 as described in Example 1 and the resulting strain was named yEA108.

B)

The following Polymerase Chain Reaction (PCR) was performed using the Gene Amp PCR reagent kit (Perkin Elmer, 761 Main Avewalk, CT 06859, USA) according to the manufacturer's instructions. In all cases, the PCR mixture was overlayed with 100 µl of mineral oil (Sigma Chemical Co., St. Louis, MO, USA)

5 µl of oligonucleotide #220 (100 pmol)
5 µl of oligonucleotide #307 (100 pmol)
10 µl of 10X PCR buffer
16 µl of dNTP mix
0.5 µl of Taq enzyme
0.2 µl of pEA108 plasmid as template (0.1 ug DNA)
63 µl of water

A total of 16 cycles were performed, each cycle comprising 1 minute at 95°C; 1 minute at 40°C; and 2 minutes at 72°C. The PCR mixture was then loaded onto an 2% agarose gel and subjected to electrophoresis using standard techniques. The resulting DNA fragment was cut out of the agarose gel and isolated using the Gene Clean kit (Bio 101 Inc., PO BOX 2284, La Jolla, CA 92038, USA) according to the manufacture's instructions. The purified PCR DNA fragment was dissolved in 10 µl of water and restriction endonuclease buffer and cut with the restriction endonucleases NcoI and XbaI according to standard techniques. The NcoI/XbaI DNA fragment was purified using The Gene Clean Kit as described.

The plasmid pAK401 consists of a DNA sequence of 523 bp composed of an EcoRI/NcoI fragment derived from pMT636 (described in WO 90/10075) (constructed by by introducing a NcoI site in the 3'-end of the alpha leader by site directed mutagenesis) encoding the alpha factor leader followed by a NcoI/XbaI fragment from pAK188 encoding the insulin precursor MI5 (see SEQ ID NOS. 41, 42 and 43) inserted into the vector (phagemid) pBLUESCRIPT IIsk(+/-) (Stratagene, USA). The plasmid pAK401 is shown in Fig. 3.

The plasmid pAK401 was cut with the restriction endonucleases NcoI and XbaI and the vector fragment of 3254 bp isolated and ligated together with the NcoI/XbaI PCR fragment. The ligation mixture was then transformed into a competent E. coli strain and plasmids were isolated from the resulting E. coli colonies using a standard DNA miniprep technique and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI. The selected plasmid, named p113A (shown in Fig. 3), was cut with EcoRI and XbaI and the fragment of 535 bp isolated.

The plasmid pAK233 was cut with the restriction endonucleases NcoI and XbaI, and with EcoRI/NcoI and the fragments of 9273 and 1644 bp isolated. These two DNA fragments were ligated together with the EcoRI/XbaI fragment from p113A using T4 DNA

ligase and standard conditions. The ligation mixture was then transformed into a competent E. coli strain (R-, M+) followed by selection for ampicillin resistance. Plasmids were isolated from the resulting E. coli colonies using a standard DNA miniprep technique and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI, HindIII. The selected plasmid was shown by DNA sequencing analyses to contain the correct sequence for the Arg^{B31} single chain human insulin precursor DNA with the N-terminal extension GluGluAlaGluAlaGluAlaArg and to be inserted after the DNA encoding the S. cerevisiae alpha factor DNA. The plasmid was named pEA113 and is shown in Fig. 3. The DNA sequence encoding the alpha factor leader/Arg^{B-1} Arg^{B31} single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaArg) and the amino acid sequence thereof are SEQ ID NOS. 44, 45 and 46. The plasmid pEA113 was transformed into S. cerevisiae strain MT663 as described in Example 1 and the resulting strain was named yEA113.

EXAMPLE 6

Synthesis of Arg^{B-1} Arg^{B31} single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaGluArg) from Yeast strain yEA136 using the alpha factor leader.

The following oligonucleotide was synthesized:

#389 5'-GCTAACGTCGCCATGGCTAAGAGAGAAGAAGCTGAAGCGAAG CTGAAAGATTCGTTAACCAACAC-3' (SEQ ID NO:13)

The following PCR was performed using the Gene Amp PCR reagent kit

5 µl of oligonucleotide #220 (100 pmol)
5 µl of oligonucleotide #389 (100 pmol)
10 µl of 10X PCR buffer

16 µl of dNTP mix
0.5 µl of Taq enzyme
2 µl of pEA113 plasmid as template (0.5 ug DNA)
63 µl of water

A total of 12 cycles were performed, each cycle comprising 1 minute at 95°C; 1 minute at 37°C; and 2 minutes at 72°C.

The DNA encoding alpha factor leader/Arg^{B-1} Arg^{B31} single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaGluArg) was constructed in the same manner as described for the DNA encoding alpha factor leader/Arg^{B-1} Arg^{B31} single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaArg) in Example 5. The plasmid was named pEA136. The DNA sequence encoding the alpha factor leader/Arg^{B-1} Arg^{B31} single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaGluArg) and the amino acid sequence thereof are SEQ ID NOS. 47, 48 and 49. The plasmid pEA136 was transformed into S. cerevisiae strain MT663 as described in Example 1 and the resulting strain was named yEA136.

EXAMPLE 7

Synthesis of (A1,B1)-diBoc human insulin.

---

5 g of zinc-free human insulin was dissolved in 41.3 ml of DMSO. To the solution was added 3.090 ml of acetic acid. The reaction was conducted at room temperature and initiated by addition of 565 mg of di-tert-butyl pyrocarbonate dissolved in 5.650 ml of DMSO. The reaction was allowed to proceed for 5½ hour and then stopped by addition of 250 µl of ethanolamine. The product was precipitated by addition of 1500 ml of acetone. The precipitate was isolated by centrifugation and dried in vacuum. A yield of 6.85 g material was obtained.

(A1,B1)-diBoc insulin was purified by reversed phase HPLC as follows: The crude product was dissolved in 100 ml of 25% ethanol in water, adjusted to pH 3.0 with HCl and applied to a column (5 cm diameter, 30 cm high) packed with octadecyldimethylsilyl-substituted silica particles (mean particle size 15 μm, pore size 100 Å) and equilibrated with elution buffer. The elution was performed using mixtures of ethanol and 1 mM aqueous HCl, 0.3 M KCl at a flow of 2 l/h. The insulin was eluted by increasing the ethanol content from 30% to 45%. The appropriate fraction was diluted to 20% ethanol and precipitated at pH 4.8. The precipitated material was isolated by centrifugation and dried in vacuum. Thus 1.701 g of (A1,B1)-diBoc human insulin was obtained at a purity of 94.5%.

EXAMPLE 8

Synthesis of $(N^{\epsilon B29}$-benzoyl human insulin$)_6$, $3Zn^{2+}$.

---

400 mg of (A1,B1)-diBoc human insulin was dissolved in 2 ml of DMSO. To the solution was added 748 μl of a mixture of N-methylmorpholine and DMSO (1:9, v/v). The reaction was conducted at 15°C and initiated by addition of 14.6 mg of benzoic acid N-hydroxysuccinimide ester dissolved in 132 μl DMF. The reaction was stopped after 2 hours by addition of 100 ml of acetone. The precipitated material was isolated by centrifugation and dried in vacuum. 343 mg of material was collected.

The Boc protecting groups were eliminated by addition of 4 ml of TFA. The dissolved material was incubated for 30 minutes and then precipitated by addition of 50 ml of acetone. The precipitate was isolated by centrifugation and dried in vacuum.

$N^{\epsilon B29}$-benzoyl human insulin was purified by reversed phase HPLC as described in Example 7. A yield of 230 mg was obtained. Recrystallization from 15% aqueous ethanol containing 6 mM Zn^{2+}

and 50 mM citrate at pH 5.5 gave crystals of the title compound which were isolated by centrifugation and dried in vacuum. The yield was 190 mg.

Molecular mass, found by MS: 5911, theory: 5911.

EXAMPLE 9

Synthesis of ($N^{\epsilon B29}$-lithocholoyl human insulin)$_6$, $3Zn^{2+}$.

---

400 mg of (A1,B1)-diBoc human insulin was dissolved in 2 ml of DMSO. To the solution was added 748 µl of a mixture of N-methylmorpholine and DMSO (1:9, v/v). The reaction was conducted at 15°C and initiated by addition of 31.94 mg of lithocholic acid N-hydroxysuccinimide ester dissolved in 300 µl of DMF. The reaction was stopped after 2 hours by addition of 100 ml of acetone. The precipitated material was isolated by centrifugation and dried in vacuum. 331 mg of material was obtained.

The Boc protecting groups were eliminated by addition of 4 ml of TFA. The dissolved material was incubated for 30 minutes and then precipitated by addition of 50 ml of acetone. The precipitate was isolated by centrifugation and dried in vacuum. The yield was 376 mg.

B29-lithocholoyl insulin was purified by reversed phase HPLC as described in Example 7. A final yield of 67 mg was obtained at a purity of 94%. Recrystallization from 15% aqueous ethanol containing 6 mM Zn^{2+} and 50 mM citrate at pH 5.5 gave crystals of the title compound which were isolated by centrifugation and dried in vacuum. The yield was 49 mg.

Molecular mass, found by MS: 6160, theory: 6166.

EXAMPLE 10

Synthesis of ($N^{\epsilon B29}$-decanoyl human insulin)$_6$, $3Zn^{2+}$.

---

400 mg of (A1,B1)-diBoc human insulin was dissolved in 2 ml of DMSO. To the solution was added 748 µl of a mixture of N-methylmorpholine and DMSO (1:9, v/v). The reaction was conducted at 15°C and initiated by addition of 18.0 mg of decanoic acid N-hydroxysuccinimide ester dissolved in 132 µl of DMF. The reaction was stopped after 60 minutes and the product precipitated by addition of 100 ml of acetone. The precipitated material was isolated by centrifugation and dried in vacuum. 420 mg of intermediate product was collected.

The Boc protecting groups were eliminated by addition of 4 ml of TFA. The dissolved material was incubated for 30 minutes and the product was then precipitated by addition of 50 ml of acetone. The precipitate was isolated by centrifugation and dried in vacuum. The yield of crude product was 420 mg.

The crude product was purified by reversed phase HPLC as described in Example 7. A final yield of 254 mg of the title product was obtained. The purity was 96.1%. Recrystallization from 15% aqueous ethanol containing 6 mM Zn^{2+} and 50 mM citrate at pH 5.5 gave crystals of the title compound which were isolated by centrifugation and dried in vacuum. The yield was 217 mg.

Molecular mass, found by MS: 5962, theory: 5962.

EXAMPLE 11

Synthesis of des(B30) human insulin.

Synthesis of des(B30) human insulin was carried out as described by Markussen (Methods in diabetes research, Vol. I,

Laboratory methods, part B, 404-410. Ed: J. Larner and S. Phol, John Wiley & Sons, 1984). 5 g of human insulin was dissolved in 500 ml of water while the pH value of the solution was kept at 2.6 by addition of 0.5 M sulphuric acid. Subsequently, the insulin was salted out by addition of 100 g of ammonium sulphate and the precipitate was isolated by centrifugation. The pellet was dissolved in 800 ml of 0.1 M ammonium hydrogen carbonate and the pH value of the solution was adjusted to 8.4 with 1 M ammonia.

50 mg of bovine carboxypeptidase A was suspended in 25 ml of water and isolated by centrifugation. The crystals were suspended in 25 ml of water and 1 M ammonia was added until a clear solution was obtained at a final pH of 10. The carboxypeptidase solution was added to the insulin solution and the reaction was allowed to proceed for 24 hours. A few drops of toluene were added to act as preservative during the reaction.

After 24 hours the des(B30) human insulin was crystallized by successive addition of 80 g of sodium chloride while the solution was stirred. The pH value was then adjusted to 8.3 and the crystallization was allowed to proceed for 20 hours with gentle stirring. The crystals were isolated on a 1.2 µm filter, washed with 250 ml of ice cold 2-propanol and finally dried in vacuum.

EXAMPLE 12

Synthesis of (A1,B1)-diBoc des(B30) human insulin.

The title compound was synthesized by a method similar to that described in Example 7, using des(B30) porcine insulin as the starting material. The crude product was precipitated by acetone and dried in vacuum. The (A1,B1)-diBoc des(B30) human

insulin was purified by reversed phase HPLC as described in Example 7.

EXAMPLE 13

Synthesis of $N^{\epsilon B29}$-decanoyl des(B30) human insulin.

400 mg of (A1,B1)-diBoc des(B30) human insulin was used as starting material for the synthesis of $N^{\epsilon B29}$-decanoyl des(B30) human insulin, following the procedure described in Example 10. The crude product was precipitated by acetone, dried in vacuum and deprotected using TFA. The resulting product was precipitated by acetone and dried in vacuum. $N^{\epsilon B29}$-decanoyl des(B30) human insulin was then purified by reversed phase HPLC as described in Example 10.

Molecular mass, found by MS: 5856, theory: 5861.

EXAMPLE 14

Synthesis of $N^{\epsilon B29}$-dodecanoyl des(B30) human insulin.

a. Immobilization of A. lyticus protease

13 mg of A. lyticus protease, dissolved in 5 ml of aqueous 0.2 M $NaHCO_3$ buffer, pH 9.4, was mixed with 4 ml of settled MiniLeak® Medium gel, which had been washed with the same buffer (MiniLeak is a divinylsulfone activated Sepharose CL 6B, obtained from KemEnTec, Copenhagen). The gel was kept in suspension by gentle stirring for 24 hours at room temperature. Then, the gel was isolated by filtration, washed with water, and suspended in 20 ml of 1 M ethanolamine buffer, pH 9.4, and kept in suspension for 24 hours at room temperature. Finally, the gel was washed with water followed by 0.1 M acetic acid and stored at 4°C. The enzyme activity in the filtrate was 13% of

that in the initial solution, indicating a yield in the immobilization reaction of about 87%.

b. Immobilization of porcine trypsin

Porcine trypsin was immobilized to MiniLeak® Low to a degree of substitution of 1 mg per ml of gel, using the conditions described above for immobilization of A. lyticus.

c. Synthesis of Glu(GluAla)$_3$Arg-B(1-29), ThrArg-A(1-21) insulin using immobilized A. lyticus protease

To 200 mg of Glu(GluAla)$_3$Arg-B(1-29)-ThrArg-A(1-21) single-chain human insulin precursor, dissolved in 20 ml of 0.1 M $NaHCO_3$ buffer, pH 9.0, was added 4 ml of the gel carrying the immobilized A. lyticus protease. After the gel had been kept in suspension in the reaction mixture for 6 hours at room temperature the hydrolysis was complete, rendering Glu(GluAla)$_3$-Arg-B(1-29), ThrArg-A(1-21) human insulin (the reaction was followed by reversed phase HPLC). After the hydrolysis, the gel was removed by filtration. To the filtrate was added 5 ml of ethanol and 15 µL of 1 M $ZnCl_2$ and the pH was adjusted to 5.0 using HCl. The precipitation of the product was completed on standing overnight at 4°C with gentle stirring. The product was isolated by centrifugation. After one washing with 1 ml of ice cold 20% ethanol and drying in vacuo the yield was 190 mg.

d. Synthesis of $N^{\alpha A1}$,$N^{\alpha B1}$,$N^{\epsilon B29}$-tridodecanoyl Glu(GluAla)$_3$Arg-B(1-29), Thr-Arg-A(1-21) human insulin using dodecanoic acid N-hydroxysuccinimide ester

190 mg (30 µmol) of Glu(GluAla)$_3$Arg-B(1-29), ThrArg-A(1-21) insulin was dissolved in 1 ml of DMSO and 1.05 ml of a 0.572 M solution of N,N-diisopropylethylamine in DMF. The solution was cooled to 15°C and 36 mg (120 µmol) of dodecanoic acid N-hydroxysuccinimide ester dissolved in 0.6 ml of DMSO was added.

The reaction was completed within 24 hours. The lipophilic title compound was not isolated.

e. Synthesis of $N^{\epsilon B29}$-dodecanoyl des(B30) insulin

The product from the previous step, d., contained in approximately 2,65 ml of DMSO/DMF/N,N-diisopropylethylamine was diluted with 10.6 ml of a 50 mM glycine buffer comprising 20% ethanol and the pH adjusted to 10 with NaOH. After standing for 1 hour at room temperature 1 ml of MiniLeak gel, carrying 1 mg of immobilized trypsin per ml of gel, was added. The reaction mixture was stirred gently for 48 hours at room temperature. In order to isolate the desired product, the reaction mixture was applied to a reversed phase HPLC column (5 cm in diameter, 30 cm high), packed with octadecyldimethylsilyl-substituted silica particles (mean particle size 15 μm, pore size 100 Å). For the elution was used 20 mM Tris/HCl buffers, adjusted to pH 7.7 and comprising an increasing concentration of ethanol, from 40% to 44% (v/v), at a rate of 2000 ml/h. The major peak eluting at about 43-44% of ethanol contained the title compound. The fractions containing the major peak were pooled, water was added to reduce the ethanol concentration to 20% (v/v), and the pH was adjusted to 5.5. The solution was left overnight at -20°C, whereby the product precipitated. The precipitate was isolated by centrifugation at -8°C and dried in vacuo. The yield of the title compound was 90 mg.

Molecular mass, found by MS: 5892, theory: 5890.

EXAMPLE 15

Synthesis of $N^{\epsilon B29}$-(N-myristoyl-α-glutamyl) human insulin.

---

500 mg of (A1,B1)-diBoc human insulin was dissolved in 2.5 ml of DMSO and 428 μl of ethyl diisopropylamine, diluted with 2.5 ml of DMSO/DMF 1/1 (v/v), was added. The temperature was

adjusted to 15°C and 85 mg of N-myristoyl-Glu(OBut) N-hydroxysuccinimide ester, dissolved in 2.5 ml of DMSO/DMF 1/1 (v/v), was added. After 30 min the reaction mixture was poured into 60 ml of water, the pH adjusted to 5 and the precipitate isolated by centrifugation. The precipitate was dried in vacuo. The dried reaction mixture was dissolved in 25 ml of TFA, and the solution was left for 30 min at room temperature. The TFA was removed by evaporation in vacuo. The gelatinous residue was dissolved in 60 ml of water and the pH was adjusted to 11.2 using concentrated ammonia. The title compound was crystallized from this solution by adjustment of the pH to 8.5 using 6 N HCl. The product was isolated by centrifugation, washed once by 10 ml of water, and dried in vacuo. Yield 356 mg. Purity by HPLC 94%.

The product of this example is thus human insulin wherein the ϵ-amino group of Lys^{B29} has a substituent of the following structure: $CH_3(CH_2)_{12}CONHCH(CH_2CH_2COOH)CO-$.

Molecular mass, found by MS: 6146, theory: 6148.

EXAMPLE 16

Synthesis of $N^{\epsilon B29}$-undecanoyl des(B30) human insulin.

The title compound was synthesized analogously to $N^{\epsilon B29}$-dodecanoyl des(B30) human insulin as described in Example 14, by using undecanoic acid N-hydroxysuccinimide ester instead of dodecanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 5876, theory: 5876.

EXAMPLE 17

Synthesis of $N^{\epsilon B29}$-tridecanoyl des(B30) human insulin.

The title compound was synthesized analogously to $N^{\epsilon B29}$-dodecanoyl des(B30) human insulin as described in Example 14, by using tridecanoic acid N-hydroxysuccinimide ester instead of dodecanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 5899, theory: 5904.

EXAMPLE 18

Synthesis of $N^{\epsilon B29}$-myristoyl des(B30) human insulin.

The title compound was synthesized analogously to $N^{\epsilon B29}$-dodecanoyl des(B30) human insulin as described in Example 14, by using myristic acid N-hydroxysuccinimide ester instead of dodecanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 5923, theory: 5918.

EXAMPLE 19

Synthesis of $N^{\epsilon B29}$-palmitoyl des(B30) human insulin.

The title compound was synthesized analogously to $N^{\epsilon B29}$-dodecanoyl des(B30) human insulin as described in Example 14, by using palmitic acid N-hydroxysuccinimide ester instead of dodecanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 5944, theory: 5946.

EXAMPLE 20

Synthesis of $N^{\epsilon B29}$-suberoyl-D-thyroxine human insulin.

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a. Preparation of N-(succinimidylsuberoyl)-D-thyroxine.

Disuccinimidyl suberate (1.0 g, Pierce) was dissolved in DMF (50 ml), and D-thyroxine (2.0 g, Aldrich) was added with stirring at 20°C. The thyroxine slowly dissolved, and after 20 hours the solvent was removed by evaporation in vacuo. The oily residue was crystallized from 2-propanol to yield 0.6 g of N-(succinimidylsuberoyl)-D-thyroxine, m.p. 128-133°C.

b. Reaction of (A1,B1)-diBoc human insulin with N-(succinimidylsuberoyl)-D-thyroxine.

(A1,B1)-diBoc human insulin (200 mg) was dissolved in dry DMF (10 ml) by addition of triethylamine (20 µl) at room temperature. Then, N-(succinimidylsuberoyl)-D-thyroxine (80 mg) was added. The reaction was monitored by reversed phase HPLC and when the reaction was about 90% complete, the solvent was removed in vacuo. To the evaporation residue, anhydrous trifluoroacetic acid (5 ml) was added, and the solution was kept for 1 hour at room temperature. After removal of the trifluoroacetic acid in vacuo, the residue was dissolved in a mixture of 1M acetic acid (5 ml) and acetonitrile (1.5 ml), purified by preparative reversed phase HPLC and desalted on a PD-10 column. The yield of $N^{\epsilon B29}$-suberoyl-D-thyroxine human insulin was 50 mg.

The product of this example is thus human insulin wherein the ϵ-amino group of Lys^{B29} has a substituent of the following structure: Thyrox-$CO(CH_2)_6CO$-, wherein Thyrox is thyroxine which is bound to the octanedioic acid moiety via an amide bond to its α-amino group.

Molecular mass of the product found by MS: 6724, theory: 6723.

EXAMPLE 21

Synthesis of $N^{\epsilon B29}$-(2-succinylamido)myristic acid human insulin.

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a. Preparation of α-aminomyristic acid methyl ester,HCl.

To methanol (5 ml, Merck) at -10°C, thionyl chloride (0.2 ml, Aldrich) was added dropwise while stirring vigorously. Then, α-aminomyristic acid (0.7 g, prepared from the α-bromo acid by reaction with ammonia) was added. The reaction mixture was stirred at room temperature overnight, and then evaporated to dryness. The crude product (0.7 g) was used directly in step b.

b. Preparation of N-succinoyl-α-aminomyristic acid methyl ester.

α-Aminomyristic acid methyl ester,HCl (0.7 g) was dissolved in chloroform (25 ml, Merck). Triethylamine (0.35 ml, Fluka) was added, followed by succinic anhydride (0.3 g, Fluka). The reaction mixture was stirred at room temperature for 2 hours, concentrated to dryness, and the residue recrystallized from ethyl acetate/petroleum ether (1/1). Yield: 0.8 g.

c. Preparation of N-(succinimidylsuccinoyl)-α-aminomyristic acid methyl ester.

N-succinoyl-α-aminomyristic acid methyl ester (0.8 g) was dissolved in dry DMF (10 ml, Merck, dried over 4Å molecular sieve). Dry pyridine (80 µl, Merck), and di(N-succinimidyl)carbonate (1.8 g, Fluka) were added, and the reaction mixture was stirred overnight at room temperature. The evaporation residue was purified by flash chromatography on silica gel 60 (Merck), and recrystallized from 2-propanol/petroleum ether (1/1). Yield of N-(succinimidylsuccinoyl)-α-aminomyristic acid methyl ester: 0.13 g, m.p. 64-66°C.

d. Reaction of (A1,B1)-diBoc human insulin with N-(succinimidylsuccinoyl)-α-aminomyristic acid methyl ester.

The reaction was carried out as in Example 20 b., but using N-(succinimidylsuccinoyl)-α-aminomyristic acid methyl ester (16 mg) instead of N-(succinimidylsuberoyl)-D-thyroxine. After removal of the trifluoroacetic acid in vacuo, the evaporation residue was treated with 0.1M sodium hydroxide at 0°C to saponify the methyl ester. When the saponification was judged to be complete by reversed phase HPLC, the pH value in the solution was adjusted to 3, and the solution was lyophilized. After purification by preparative reversed phase HPLC and desalting on a PD-10 column, the yield of $N^{\epsilon B29}$-(2-succinylamido)myristic acid human insulin was 39 mg.

The product of this example is thus human insulin wherein the ε-amino group of Lys^{B29} has a substituent of the following structure: $CH_3(CH_2)_{11}CH(COOH)NHCOCH_2CH_2CO-$.

Molecular mass of the product found by MS: 6130, theory: 6133.

EXAMPLE 22

Synthesis of $N^{\epsilon B29}$-octyloxycarbonyl human insulin.

The synthesis was carried out as in Example 20 b., but using n-octyloxycarbonyl N-hydroxysuccinimide (9 mg, prepared from n-octyl chloroformate (Aldrich) and N-hydroxysuccinimide), instead of N-(succinimidylsuberoyl)-D-thyroxine. The yield of $N^{\epsilon B29}$-octyloxycarbonyl human insulin was 86 mg.

The product of this example is thus human insulin wherein the ε-amino group of Lys^{B29} has a substituent of the following structure: $CH_3(CH_2)_7OCO-$.

Molecular mass of the product found by MS: 5960, theory: 5964.

EXAMPLE 23

Synthesis of $N^{\epsilon B29}$-(2-succinylamido)palmitic acid human insulin.

---

a. Preparation of N-(succinimidylsuccinoyl)-α-amino palmitic acid methyl ester.

This compound was prepared as described in Example 21 a.-c., using α-amino palmitic acid instead of α-amino myristic acid.

b. Reaction of (A1,B1)-diBoc human insulin with N-(succinimidylsuccinoyl)-α-aminopalmititctic acid methyl ester.

The reaction was carried out as in Example 21 d., but using N-(succinimidylsuccinoyl)-α-aminopalmitic acid methyl ester instead of N-(succinimidylsuccinoyl)-α-aminopalmitic acid methyl ester to give $N^{\epsilon B29}$-(2-succinylamido)palmitic acid human insulin.

The product of this example is thus human insulin wherein the ε-amino group of Lys^{B29} has a substituent of the following structure: $CH_3(CH_2)_{13}CH(COOH)NHCOCH_2CH_2CO-$.

EXAMPLE 24

Synthesis of $N^{\epsilon B29}$-(2-succinylamidoethyloxy)palmitic acid human insulin.

---

a. Preparation of N-(succinimidylsuccinoyl)-2-aminoethyloxy palmitic acid methyl ester.

This compound was prepared as described in Example 21 a.-c. but using 2-aminoethyloxy palmitic acid (synthesized by the general procedure described by R. TenBrink, J. Org. Chem. 52 (1987) 418-422 instead of α-amino myristic acid.

b. Reaction of (A1,B1)-diBoc human insulin with N-(succinimidylsuccinoyl)-2-aminoethyloxypalmititctic acid methyl ester.

The reaction was carried out as in Example 21 d., but using N-(succinimidylsuccinoyl)-2-aminoethyloxypalmitic acid methyl ester instead of N-(succinimidylsuccinoyl)-α-aminomyristic acid methyl ester to give $N^{\epsilon B29}$-(2-succinylamidoethyloxy)palmitic acid human insulin.

The product of this example is thus human insulin wherein the ε-amino group of Lys^{B29} has a substituent of the following structure: $CH_3(CH_2)_{13}CH(COOH)NHCH_2CH_2OCOCH_2CH_2CO-$.

EXAMPLE 25

Synthesis of $N^{\epsilon B29}$-lithocholoyl-α-glutamyl des(B30) human insulin.

The synthesis was carried out as in Example 13 using N-lithocholoyl-L-glutamic acid α-N-hydroxysuccinimide ester, γ-tert-butyl ester instead of decanoic acid N-hydroxysuccinimide ester.

The product of this example is thus des(B30) human insulin wherein the ε-amino group of Lys^{B29} has a substituent of the following structure: lithocholoyl-$NHCH(CH_2CH_2COOH)CO-$.

Molecular mass of the product found by MS: 6194, theory: 6193.

EXAMPLE 26

Synthesis of $N^{\epsilon B29}$-3,3',5,5'-tetraiodothyroacetyl human insulin.

The synthesis was carried out as in Example 10 using 3,3',5,5'-tetraiodothyroacetic acid N-hydroxysuccinimide ester, instead of decanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 6536, theory: 6538.

EXAMPLE 27

Synthesis of $N^{\epsilon B29}$-L-thyroxyl human insulin.

The synthesis was carried out as in Example 10 using Boc-L-thyroxine N-hydroxysuccinimide ester, instead of decanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 6572, theory: 6567.

EXAMPLE 28

A pharmaceutical composition comprising 600 nmol/ml of $N^{\epsilon B29}$-decanoyl des(B30) human insulin, $1/3Zn^{2+}$ in solution.

$N^{\epsilon B29}$-decanoyl des(B30) human insulin (1.2 µmol) was dissolved in water (0.8 ml) and the pH value was adjusted to 7.5 by addition of 0.2 M sodium hydroxide. 0.01 M zinc acetate (60 µl) and a solution containing 0.75% of phenol and 4% of glycerol (0.8 ml) was added. The pH value of the solution was adjusted to 7.5 using 0.2 M sodium hydroxide and the volume of the solution was adjusted to 2 ml with water.

The resulting solution was sterilized by filtration and transferred aseptically to a cartridge or a vial.

EXAMPLE 29

A pharmaceutical composition comprising 600 nmol/ml of $N^{\epsilon B29}$-decanoyl human insulin, $\frac{1}{2}Zn^{2+}$ in solution.

1.2 μmol of the title compound was dissolved in water (0.8 ml) and the pH value was adjusted to 7.5 by addition of 0.2 M sodium hydroxide. A solution containing 0.75% of phenol and 1.75% of sodium chloride (0.8 ml) was added. The pH value of the solution was adjusted to 7.5 using 0.2 M sodium hydroxide and the volume of the solution was adjusted to 2 ml with water.

The resulting solution was sterilized by filtration and transferred aseptically to a cartridge or a vial.

EXAMPLE 30

A pharmaceutical composition comprising 600 nmol/ml of $N^{\epsilon B29}$-lithocholoyl human insulin in solution.

1.2 μmol of the title compound was suspended in water (0.8 ml) and dissolved by adjusting the pH value of the solution to 8.5 using 0.2 M sodium hydroxide. To the solution was then added 0.8 ml of a stock solution containing 0.75 % cresol and 4% glycerol in water. Finally, the pH value was again adjusted to 8.5 and the volume of the solution was adjusted to 2 ml with water.

The resulting solution was sterilized by filtration and transferred aseptically to a cartridge or a vial.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Novo Nordisk A/S
(B) STREET: Novo Allé
(C) CITY: DK-2880 Bagsvaerd
(E) COUNTRY: Denmark
(G) TELEPHONE: +45 44448888
(H) TELEFAX: +45 44490555
(I) TELEX: 37173

(ii) TITLE OF INVENTION: ACYLATED INSULIN

(iii) NUMBER OF SEQUENCES: 49

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Novo Nordisk A/S
Corporate Patents
(B) STREET: Novo Alle
(C) CITY: DK-2880 Bagsvaerd
(E) COUNTRY: Denmark

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBERS: DK 1044/93 and US 08/190,829
(B) FILING DATES: 09-SEP-1993 and 02-FEB-1994

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Jørgensen, Dan et al.
(C) REFERENCE/DOCKET NUMBER: 3985.204-WO,DJ

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: +45 44448888
(B) TELEFAX: +45 44493256

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu
1 5 10 15

Glu Asn Tyr Cys Xaa
20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Xaa Val Xaa Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr
1 5 10 15

Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Xaa
20 25 30

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGGCTAAGAG ATTCGTTGAC CAACACTTGT GCGGTTCTCA CTTGGTTGAA GCTTTGTACT 60

TGGTTTGTGG TGAAAGAGGT TTCTTCTACA CTCCAAAGTC TGACGACGCT 110

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 100 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTGCGGGCTG CGTCTAAGCA CAGTAGTTTT CCAATTGGTA CAAAGAACAG ATAGAAGTAC 60

AACATTGTTC AACGATACCC TTAGCGTCGT CAGACTTTGG 100

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTCGCCATGG CTAAGAGATT CGTTG 25

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTGCTCTAGA GCCTGCGGGC TGCGTCT 27

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TGGCTAAGAG ATTCGTTACT CAACACTTGT GCGGTTCTCA CTTGGTTGAA GCTTTGTACT 60

TGGTTTGTGG TGAAAGAGGT TTCTTCTACA CTCCAAAGTC TGACGACGCT 110

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTCGCCATGG CTAAGAGATT CGTTA 25

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 100 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTGCGGGCTG CGTCTAACCA CAGTAGTTTT CCAATTGGTA CAAAGAACAG ATAGAAGTAC 60

AACATTGTTC AACGATACCC TTAGCGTCGT CAGACTTTGG 100

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ACGTACGTTC TAGAGCCTGC GGGCTGC 27

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 78 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CACTTGGTTG AAGCTTTGTA CTTGGTTTGT GGTGAAAGAG GTTTCTTCTA CACTCCAAAG 60

ACTAGAGGTA TCGTTGAA 78

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCTAACGTCG CCATGGCTAA GAGAGAAGAA GCTGAAGCTG AAGCTAGATT CGTTAACCAA 60

CAC 63

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCTAACGTCG CCATGGCTAA GAGAGAAGAA GCTGAAGCGA AGCTGAAAGA TTCGTTAACC 60

AACAC 65

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 415 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACTATCAA TTTCATACAC      60

AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC      112
                     Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
                       1               5                  10

GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG       160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
            15                  20                  25

ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC       208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
        30                  35                  40

GTC GCC ATG GCT AAG AGA TTC GTT AAC CAA CAC TTG TGC GGT TCT CAC       256
Val Ala Met Ala Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His
    45                  50                  55

TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC       304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
60                  65                  70                  75

ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT       352
Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr
                80                  85                  90

TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT AAC TAGACGCAGC       401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
            95                  100

CCGCAGGCTC TAGA                                                     415
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 104 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 1               5                  10                  15

Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
            20                  25                  30

Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
        35                  40                  45

Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
    50                  55                  60
```

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
65 70 75 80

Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
85 90 95

Tyr Gln Leu Glu Asn Tyr Cys Asn
100

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 415 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG 60

TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC 120

GACCCGGGTT GGTCAGTGAC CGCTACTTAG TAGACAACTC TAAGGCCTTC TCAGAGACTA 180

GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AATTGGTTGT 240

GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA 300

GATGTGAGGT TTCAGACTGC TGCGATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC 360

AAGAAACATG GTTAACCTTT TGATGACATT GATCTGCGTC GGGCGTCCGA GATCT 415

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 523 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 80..499

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACTATCAA TTTCATACAC 60

```
AATATAAACG ATTAAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA      112
                      Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
                        1               5                  10

TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA      160
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu
            15                  20                  25

GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT      208
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp
        30                  35                  40

TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA      256
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr
    45                  50                  55

AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT      304
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala
60                  65                  70                  75

AAA GAA GAA GGG GTA TCT TTG GAT AAG AGA GAA GTT AAC CAA CAC TTG      352
Lys Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Asn Gln His Leu
                80                  85                  90

TGC GGT TCT CAC TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA      400
Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg
            95                  100                 105

GGT TTC TTC TAC ACT GAA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA      448
Gly Phe Phe Tyr Thr Glu Lys Ser Asp Asp Ala Lys Gly Ile Val Glu
        110                 115                 120

CAA TGT TGT ACT TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT      496
Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys
    125                 130                 135

AAC TAGACGCAGC CCGCAGGCTC TAGA                                      523
Asn
140
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 140 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
 1               5                  10                  15
```

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
20 25 30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
35 40 45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
50 55 60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
65 70 75 80

Ser Leu Asp Lys Arg Glu Val Asn Gln His Leu Cys Gly Ser His Leu
85 90 95

Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr
100 105 110

Glu Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser
115 120 125

Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
130 135 140

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 523 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG 60

TTATATTTGC TAATTTTCTT ACTCTAAAGG AAGTTAAAAA TGACGTCAAA ATAAGCGTCG 120

TAGGAGGCGT AATCGACGAG GTCAGTTGTG ATGTTGTCTT CTACTTTGCC GTGTTTAAGG 180

CCGACTTCGA CAGTAGCCAA TGAGTCTAAA TCTTCCCCTA AAGCTACAAC GACAAAACGG 240

TAAAAGGTTG TCGTGTTTAT TGCCCAATAA CAAATATTTA TGATGATAAC GGTCGTAACG 300

ACGATTTCTT CTTCCCCATA GAAACCTATT CTCTCTTCAA TTGGTTGTGA ACACGCCAAG 360

AGTGAACCAA CTTCGAAACA TGAACCAAAC ACCACTTTCT CCAAAGAAGA TGTGACTTTT 420

CAGACTGCTG CGATTCCCAT AGCAACTTGT TACAACATGA AGATAGACAA GAAACATGGT 480

TAACCTTTTG ATGACATTGA TCTGCGTCGG GCGTCCGAGA TCT 523

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 415 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACTATCAA TTTCATACAC      60

AATATAAACG ACCAAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC      112
                      Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
                        1               5                  10

GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG        160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
            15                  20                  25

ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC        208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
        30                  35                  40

GTC GCC ATG GCT AAG AGA TTC GTT GAC CAA CAC TTG TGC GGT TCT CAC        256
Val Ala Met Ala Lys Arg Phe Val Asp Gln His Leu Cys Gly Ser His
    45                  50                  55

TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC        304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
60                  65                  70                  75

ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT        352
Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr
                80                  85                  90

TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GCT TAGACGCAGC        401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Ala
            95                  100

CCGCAGGCTC TAGA                                                      415
```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 104 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 1               5                  10                  15

Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
            20                  25                  30

Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
        35                  40                  45

Arg Phe Val Asp Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
    50                  55                  60

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
65                  70                  75                  80

Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
                85                  90                  95

Tyr Gln Leu Glu Asn Tyr Cys Ala
            100
```

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 415 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG     60

TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC    120

GACCCGGGTT GGTCAGTGAC CGCTACTTAG TAGACAACTC TAAGGCCTTC TCAGAGACTA    180

GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AACTGGTTGT    240

GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA    300

GATGTGAGGT TTCAGACTGC TGCGATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC    360

AAGAAACATG GTTAACCTTT TGATGACACG AATCTGCGTC GGGCGTCCGA GATCT         415
```

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 415 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACTATCAA TTTCATACAC      60

AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC      112
                     Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
                       1               5                  10

GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG       160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
            15                  20                  25

ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC       208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
        30                  35                  40

GTC GCC ATG GCT AAG AGA TTC GTT ACT CAA CAC TTG TGC GGT TCT CAC       256
Val Ala Met Ala Lys Arg Phe Val Thr Gln His Leu Cys Gly Ser His
    45                  50                  55

TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC       304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
60                  65                  70                  75

ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT       352
Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr
                80                  85                  90

TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GCT TAGACGCAGC       401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Ala
            95                  100

CCGCAGGCTC TAGA                                                      415
```

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 104 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 1               5                  10                  15

Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
            20                  25                  30

Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
        35                  40                  45

Arg Phe Val Thr Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
    50                  55                  60

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
65                  70                  75                  80

Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
                85                  90                  95

Tyr Gln Leu Glu Asn Tyr Cys Ala
            100
```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 415 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```
TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG     60

TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC    120

GACCCGGGTT GGTCAGTGAC CGCTACTTAG TAGACAACTC TAAGGCCTTC TCAGAGACTA    180

GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AATGAGTTGT    240

GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA    300

GATGTGAGGT TTCAGACTGC TGCGATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC    360

AAGAAACATG GTTAACCTTT TGATGACACG AATCTGCGTC GGGCGTCCGA GATCT         415
```

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 415 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```
ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACTATCAA TTTCATACAC      60

AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC      112
                     Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
                       1               5                  10

GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG       160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
            15                  20                  25

ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC       208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
        30                  35                  40

GTC GCC ATG GCT AAG AGA TTC GTT GAC CAA CAC TTG TGC GGT TCT CAC       256
Val Ala Met Ala Lys Arg Phe Val Asp Gln His Leu Cys Gly Ser His
    45                  50                  55

TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC       304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
60                  65                  70                  75

ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT       352
Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr
                80                  85                  90

TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GGT TAGACGCAGC       401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Gly
            95                  100

CCGCAGGCTC TAGA                                                      415
```

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 104 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

```
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
  1               5                  10                  15
```

```
Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
            20                  25                  30

Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
        35                  40                  45

Arg Phe Val Asp Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
    50                  55                  60

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
65                  70                  75                  80

Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
                85                  90                  95

Tyr Gln Leu Glu Asn Tyr Cys Gly
            100
```

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 415 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

```
TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG     60

TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC    120

GACCCGGGTT GGTCAGTGAC CGCTACTTAG TAGACAACTC TAAGGCCTTC TCAGAGACTA    180

GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AACTGGTTGT    240

GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA    300

GATGTGAGGT TTCAGACTGC TGCGATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC    360

AAGAAACATG GTTAACCTTT TGATGACACC AATCTGCGTC GGGCGTCCGA GATCT         415
```

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 415 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

```
ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACTATCAA TTTCATACAC      60

AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC      112
                     Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
                       1               5                  10

GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG       160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
            15                  20                  25

ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC       208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
        30                  35                  40

GTC GCC ATG GCT AAG AGA TTC GTT ACT CAA CAC TTG TGC GGT TCT CAC       256
Val Ala Met Ala Lys Arg Phe Val Thr Gln His Leu Cys Gly Ser His
    45                  50                  55

TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC       304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
60                  65                  70                  75

ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT       352
Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr
                80                  85                  90

TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GGT TAGACGCAGC       401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Gly
            95                  100

CCGCAGGCTC TAGA                                                     415
```

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 104 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

```
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 1               5                  10                  15

Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
            20                  25                  30
```

Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
35 40 45

Arg Phe Val Thr Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
50 55 60

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
65 70 75 80

Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
85 90 95

Tyr Gln Leu Glu Asn Tyr Cys Gly
100

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 415 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG 60

TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC 120

GACCCGGGTT GGTCAGTGAC CGCTACTTAG TAGACAACTC TAAGGCCTTC TCAGAGACTA 180

GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AATGAGTTGT 240

GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA 300

GATGTGAGGT TTCAGACTGC TGCGATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC 360

AAGAAACATG GTTAACCTTT TGATGACACC AATCTGCGTC GGGCGTCCGA GATCT 415

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 523 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS

(B) LOCATION: 80..499

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

```
ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACTATCAA TTTCATACAC      60

AATATAAACG ATTAAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA      112
                     Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
                       1               5                  10

TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA      160
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu
            15                  20                  25

GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT      208
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp
        30                  35                  40

TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA      256
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr
    45                  50                  55

AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT      304
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala
60                  65                  70                  75

AAA GAA GAA GGG GTA TCT TTG GAT AAG AGA TTC GTT AAC CAA CAC TTG      352
Lys Glu Glu Gly Val Ser Leu Asp Lys Arg Phe Val Asn Gln His Leu
                80                  85                  90

TGC GGT TCT CAC TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA      400
Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg
            95                  100                 105

GGT TTC TTC TAC ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA      448
Gly Phe Phe Tyr Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu
        110                 115                 120

CAA TGT TGT ACT TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT      496
Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys
    125                 130                 135

AAC TAGACGCAGC CCGCAGGCTC TAGA                                      523
Asn
140
```

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 140 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

```
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
 1               5                  10                  15

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
            20                  25                  30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
        35                  40                  45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
    50                  55                  60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
65                  70                  75                  80

Ser Leu Asp Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu
                85                  90                  95

Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr
            100                 105                 110

Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser
        115                 120                 125

Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
    130                 135                 140
```

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 523 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

```
TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG     60

TTATATTTGC TAATTTTCTT ACTCTAAAGG AAGTTAAAAA TGACGTCAAA ATAAGCGTCG    120

TAGGAGGCGT AATCGACGAG GTCAGTTGTG ATGTTGTCTT CTACTTTGCC GTGTTTAAGG    180

CCGACTTCGA CAGTAGCCAA TGAGTCTAAA TCTTCCCCTA AAGCTACAAC GACAAAACGG    240

TAAAAGGTTG TCGTGTTTAT TGCCCAATAA CAAATATTTA TGATGATAAC GGTCGTAACG    300

ACGATTTCTT CTTCCCCATA GAAACCTATT CTCTAAGCAA TTGGTTGTGA ACACGCCAAG    360

AGTGAACCAA CTTCGAAACA TGAACCAAAC ACCACTTTCT CCAAAGAAGA TGTGAGGTTT    420

CAGACTGCTG CGATTCCCAT AGCAACTTGT TACAACATGA AGATAGACAA GAAACATGGT    480
```

TAACCTTTTG ATGACATTGA TCTGCGTCGG GCGTCCGAGA TCT 523

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 409 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 80..385

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

```
ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACTATCAA TTTCATACAC      60

AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC      112
                     Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
                       1               5                  10

GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG      160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
            15                  20                  25

ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC      208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
        30                  35                  40

GTC GCC ATG GCT AAG AGA TTC GTT AAC CAA CAC TTG TGC GGT TCT CAC      256
Val Ala Met Ala Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His
    45                  50                  55

TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC      304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
60                  65                  70                  75

ACT CCT AAG GAA AAG AGA GGT ATC GTT GAA CAA TGT TGT ACT TCT ATC      352
Thr Pro Lys Glu Lys Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile
                80                  85                  90

TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GGT TAGACGCAGC CCGCAGGCTC     405
Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Gly
            95                  100

TAGA                                                                 409
```

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 102 amino acids

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
1 5 10 15

Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
20 25 30

Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
35 40 45

Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
50 55 60

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Glu Lys
65 70 75 80

Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln
85 90 95

Leu Glu Asn Tyr Cys Gly
100

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 409 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG 60

TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC 120

GACCCGGGTT GGTCAGTGAC CGCTACTTAG TAGACAACTC TAAGGCCTTC TCAGAGACTA 180

GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AATTGGTTGT 240

GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA 300

GATGTGAGGA TTCCTTTTCT CTCCATAGCA ACTTGTTACA ACATGAAGAT AGACAAGAAA 360

CATGGTTAAC CTTTTGATGA CACCAATCTG CGTCGGGCGT CCGAGATCT 409

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 511 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 77..487

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

```
GAATTCCATT CAAGAATAGT TCAAACAAGA AGATTACAAA CTATCAATTT CATACACAAT      60

ATAAACGATT AAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA          109
                  Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
                    1               5                  10

TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA        157
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu
            15                  20                  25

GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT        205
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp
        30                  35                  40

TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA        253
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr
    45                  50                  55

AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT        301
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala
60                  65                  70                  75

AAA GAA GAA GGG GTA TCC ATG GCT AAG AGA TTC GTT AAC CAA CAC TTG        349
Lys Glu Glu Gly Val Ser Met Ala Lys Arg Phe Val Asn Gln His Leu
                80                  85                  90

TGC GGT TCC CAC TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA        397
Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg
            95                  100                 105

GGT TTC TTC TAC ACT CCA AAG ACT AGA GGT ATC GTT GAA CAA TGT TGT        445
Gly Phe Phe Tyr Thr Pro Lys Thr Arg Gly Ile Val Glu Gln Cys Cys
        110                 115                 120

ACT TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGC AAC                487
Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
    125                 130                 135

TAGACGCAGC CCGCAGGCTC TAGA                                            511
```

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 137 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

```
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
 1               5                  10                  15

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
            20                  25                  30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
        35                  40                  45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
    50                  55                  60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
65                  70                  75                  80

Ser Met Ala Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu
                85                  90                  95

Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr
            100                 105                 110

Pro Lys Thr Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser
        115                 120                 125

Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
    130                 135
```

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 511 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

```
CTTAAGGTAA GTTCTTATCA AGTTTGTTCT TCTAATGTTT GATAGTTAAA GTATGTGTTA      60

TATTTGCTAA TTTTCTTACT CTAAAGGAAG TTAAAAATGA CGTCAAAATA AGCGTCGTAG     120
```

GAGGCGTAAT CGACGAGGTC AGTTGTGATG TTGTCTTCTA CTTTGCCGTG TTTAAGGCCG 180

ACTTCGACAG TAGCCAATGA GTCTAAATCT TCCCCTAAAG CTACAACGAC AAAACGGTAA 240

AAGGTTGTCG TGTTTATTGC CCAATAACAA ATATTTATGA TGATAACGGT CGTAACGACG 300

ATTTCTTCTT CCCCATAGGT ACCGATTCTC TAAGCAATTG GTTGTGAACA CGCCAAGGGT 360

GAACCAACTT CGAAACATGA ACCAAACACC ACTTTCTCCA AAGAAGATGT GAGGTTTCTG 420

ATCTCCATAG CAACTTGTTA CAACATGAAG ATAGACAAGA AACATGGTTA ACCTTTTGAT 480

GACGTTGATC TGCGTCGGGC GTCCGAGATC T 511

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 523 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 80..499

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACTATCAA TTTCATACAC 60

AATATAAACG ATTAAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA 112
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
1 5 10

TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA 160
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu
15 20 25

GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT 208
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp
30 35 40

TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA 256
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr
45 50 55

AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT 304
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala
60 65 70 75

AAA GAA GAA GGG GTA TCC ATG GCT AAG AGA TTC GTT AAC CAA CAC TTG 352
Lys Glu Glu Gly Val Ser Met Ala Lys Arg Phe Val Asn Gln His Leu
80 85 90

```
TGC GGT TCC CAC TTG GTT GAA GCT TTG TAC TTG GTT TGC GGT GAA AGA      400
Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg
            95                  100                 105

GGT TTC TTC TAC ACT CCT AAG TCT GAC GAT GCT AAG GGT ATT GTC GAG      448
Gly Phe Phe Tyr Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu
        110                 115                 120

CAA TGC TGT ACC TCC ATC TGC TCC TTG TAC CAA TTG GAA AAC TAC TGC      496
Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys
    125                 130                 135

AAC TAGACGCAGC CCGCAGGCTC TAGA                                      523
Asn
140
```

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 140 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

```
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
 1               5                  10                  15

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
            20                  25                  30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
        35                  40                  45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
    50                  55                  60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
65                  70                  75                  80

Ser Met Ala Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu
                85                  90                  95

Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr
            100                 105                 110

Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser
        115                 120                 125

Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
    130                 135                 140
```

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 523 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

```
TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG      60

TTATATTTGC TAATTTTCTT ACTCTAAAGG AAGTTAAAAA TGACGTCAAA ATAAGCGTCG     120

TAGGAGGCGT AATCGACGAG GTCAGTTGTG ATGTTGTCTT CTACTTTGCC GTGTTTAAGG     180

CCGACTTCGA CAGTAGCCAA TGAGTCTAAA TCTTCCCCTA AAGCTACAAC GACAAAACGG     240

TAAAAGGTTG TCGTGTTTAT TGCCCAATAA CAAATATTTA TGATGATAAC GGTCGTAACG     300

ACGATTTCTT CTTCCCCATA GGTACCGATT CTCTAAGCAA TTGGTTGTGA ACACGCCAAG     360

GGTGAACCAA CTTCGAAACA TGAACCAAAC GCCACTTTCT CCAAAGAAGA TGTGAGGATT     420

CAGACTGCTA CGATTCCCAT AACAGCTCGT TACGACATGG AGGTAGACGA GGAACATGGT     480

TAACCTTTTG ATGACGTTGA TCTGCGTCGG GCGTCCGAGA TCT                       523
```

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 535 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 77..511

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

```
GAATTCCATT CAAGAATAGT TCAAACAAGA AGATTACAAA CTATCAATTT CATACACAAT      60

ATAAACGATT AAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA          109
                  Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
                    1               5                  10

TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA        157
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu
            15                  20                  25
```

```
GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT      205
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp
         30                  35                  40

TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA      253
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr
     45                  50                  55

AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT      301
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala
 60                  65                  70                  75

AAA GAA GAA GGG GTA TCC ATG GCT AAG AGA GAA GAA GCT GAA GCT GAA      349
Lys Glu Glu Gly Val Ser Met Ala Lys Arg Glu Glu Ala Glu Ala Glu
                 80                  85                  90

GCT AGA TTC GTT AAC CAA CAC TTG TGC GGT TCC CAC TTG GTT GAA GCT      397
Ala Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala
             95                  100                 105

TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC ACT CCA AAG ACT      445
Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr
        110                 115                 120

AGA GGT ATC GTT GAA CAA TGT TGT ACT TCT ATC TGT TCT TTG TAC CAA      493
Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln
    125                 130                 135

TTG GAA AAC TAC TGC AAC TAGACGCAGC CCGCAGGCTC TAGA                   535
Leu Glu Asn Tyr Cys Asn
140                 145
```

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 145 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

```
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
 1               5                  10                  15

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
            20                  25                  30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
        35                  40                  45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
    50                  55                  60
```

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
65 70 75 80

Ser Met Ala Lys Arg Glu Glu Ala Glu Ala Glu Ala Arg Phe Val Asn
85 90 95

Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys
100 105 110

Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr Arg Gly Ile Val Glu
115 120 125

Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys
130 135 140

Asn
145

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 535 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

CTTAAGGTAA GTTCTTATCA AGTTTGTTCT TCTAATGTTT GATAGTTAAA GTATGTGTTA 60

TATTTGCTAA TTTTCTTACT CTAAAGGAAG TTAAAAATGA CGTCAAAATA AGCGTCGTAG 120

GAGGCGTAAT CGACGAGGTC AGTTGTGATG TTGTCTTCTA CTTTGCCGTG TTTAAGGCCG 180

ACTTCGACAG TAGCCAATGA GTCTAAATCT TCCCCTAAAG CTACAACGAC AAAACGGTAA 240

AAGGTTGTCG TGTTTATTGC CCAATAACAA ATATTTATGA TGATAACGGT CGTAACGACG 300

ATTTCTTCTT CCCCATAGGT ACCGATTCTC TCTTCTTCGA CTTCGACTTC GATCTAAGCA 360

ATTGGTTGTG AACACGCCAA GGGTGAACCA ACTTCGAAAC ATGAACCAAA CACCACTTTC 420

TCCAAAGAAG ATGTGAGGTT TCTGATCTCC ATAGCAACTT GTTACAACAT GAAGATAGAC 480

AAGAAACATG GTTAACCTTT TGATGACGTT GATCTGCGTC GGGCGTCCGA GATCT 535

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 538 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 77..514

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

```
GAATTCCATT CAAGAATAGT TCAAACAAGA AGATTACAAA CTATCAATTT CATACACAAT      60

ATAAACGATT AAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA          109
                  Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
                    1               5                  10

TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA        157
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu
             15                  20                  25

GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT        205
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp
         30                  35                  40

TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA        253
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr
     45                  50                  55

AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT        301
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala
 60                  65                  70                  75

AAA GAA GAA GGG GTA TCC ATG GCT AAG AGA GAA GAA GCT GAA GCT GAA        349
Lys Glu Glu Gly Val Ser Met Ala Lys Arg Glu Glu Ala Glu Ala Glu
                 80                  85                  90

GCT GAA AGA TTC GTT AAC CAA CAC TTG TGC GGT TCC CAC TTG GTT GAA        397
Ala Glu Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu
             95                  100                 105

GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC ACT CCA AAG        445
Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys
         110                 115                 120

ACT AGA GGT ATC GTT GAA CAA TGT TGT ACT TCT ATC TGT TCT TTG TAC        493
Thr Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr
     125                 130                 135

CAA TTG GAA AAC TAC TGC AAC TAGACGCAGC CCGCAGGCTC TAGA                 538
Gln Leu Glu Asn Tyr Cys Asn
140                 145
```

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 146 amino acids

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

```
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
 1               5                  10                  15

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
            20                  25                  30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
        35                  40                  45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
    50                  55                  60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
65                  70                  75                  80

Ser Met Ala Lys Arg Glu Glu Ala Glu Ala Glu Ala Glu Arg Phe Val
                85                  90                  95

Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val
            100                 105                 110

Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr Arg Gly Ile Val
        115                 120                 125

Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr
    130                 135                 140

Cys Asn
145
```

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 538 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

```
CTTAAGGTAA GTTCTTATCA AGTTTGTTCT TCTAATGTTT GATAGTTAAA GTATGTGTTA      60

TATTTGCTAA TTTTCTTACT CTAAAGGAAG TTAAAAATGA CGTCAAAATA AGCGTCGTAG     120

GAGGCGTAAT CGACGAGGTC AGTTGTGATG TTGTCTTCTA CTTTGCCGTG TTTAAGGCCG     180
```

ACTTCGACAG TAGCCAATGA GTCTAAATCT TCCCCTAAAG CTACAACGAC AAAACGGTAA 240

AAGGTTGTCG TGTTTATTGC CCAATAACAA ATATTTATGA TGATAACGGT CGTAACGACG 300

ATTTCTTCTT CCCCATAGGT ACCGATTCTC TCTTCTTCGA CTTCGACTTC GACTTTCTAA 360

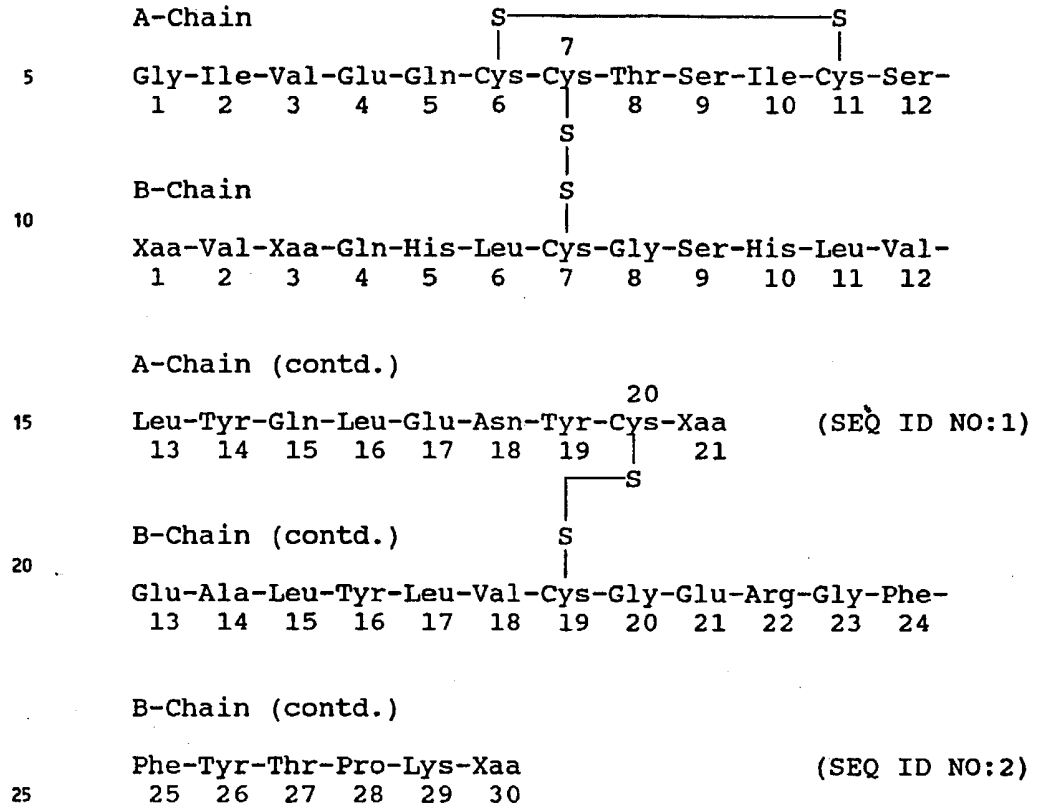
GCAATTGGTT GTGAACACGC CAAGGGTGAA CCAACTTCGA AACATGAACC AAACACCACT 420

TTCTCCAAAG AAGATGTGAG GTTTCTGATC TCCATAGCAA CTTGTTACAA CATGAAGATA 480

GACAAGAAAC ATGGTTAACC TTTTGATGAC GTTGATCTGC GTCGGGCGTC CGAGATCT 538

CLAIMS

1. An insulin derivative having the following sequence:



wherein

Xaa at positions A21 and B3 are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys;

Xaa at position B1 is Phe or is deleted;

Xaa at position B30 is (a) a non-codable, lipophilic amino acid having from 10 to 24 carbon atoms, in which case an acyl group of a carboxylic acid with up to 5 carbon atoms is bound to the ϵ-amino group of Lys^{B29}, (b) any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys, in which case the ϵ-amino group of Lys^{B29} has a lipophilic substituent or (c) deleted, in which case the ϵ-amino group of Lys^{B29} has a lipophilic substituent ; and

any Zn^{2+} complexes thereof,

provided that when Xaa at position B30 is Thr or Ala, Xaa at positions A21 and B3 are both Asn, and Xaa at position B1 is Phe, then the insulin derivative is a Zn^{2+} complex.

2. The insulin derivative according to claim 1, wherein

Xaa at positions A21 and B3 are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys;

Xaa at position B1 is Phe or is deleted;

Xaa at position B30 is a non-codable, lipophilic amino acid having from 10 to 24 carbon atoms and an acyl group is bound to the ϵ-amino group of Lys^{B29}, wherein the acyl group is an acyl group of a monocarboxylic acid with up to 4 carbon atoms or of a dicarboxylic acid with up to 5 carbon atoms.

3. The insulin derivative according to claim 1, wherein

Xaa at positions A21 and B3 are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys;

Xaa at position B1 is Phe or is deleted;

Xaa at position B30 is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys and the ϵ-amino group of Lys^{B29} has a lipophilic substituent which comprises at least 6 carbon atoms.

4. The insulin derivative according to claim 2, wherein Xaa at position B30 is selected from the group consisting of α-amino decanoic acid, α-amino dodecanoic acid, α-amino tetradecanoic acid and α-amino hexadecanoic acid.

5. The insulin derivative according to claim 2, wherein the acyl group bound to the ϵ-amino group of Lys^{B29} is selected from the group consisting of formyl, acetyl, propionyl and n-butyryl.

6. The insulin derivative according to claim 2, wherein the acyl group bound to the ϵ-amino group of Lys^{B29} is an acyl group of succinic acid.

7. The insulin derivative according to claim 3, wherein Xaa at position B30 is deleted.

8. The insulin derivative according to claim 3, wherein Xaa at position B30 is Asp, Glu, or Thr.

9. The insulin derivative according to claim 3, wherein the lipophilic substituent bound to the ϵ-amino group of Lys^{B29} is an acyl group derived from a carboxylic acid having at least 6 carbon atoms.

10. The insulin derivative according to claim 9, wherein the acyl group, which may be branched, comprises a main chain of carbon atoms 8 - 24 atoms long.

11. The insulin derivative according to claim 9, wherein the acyl group is an acyl group of a fatty acid having at least 6 carbon atoms.

12. The insulin derivative according to claim 9, wherein the acyl group is an acyl group of a linear, saturated carboxylic acid having from 6 to 24 carbon atoms.

13. The insulin derivative according to claim 9, wherein the acyl group is selected from the group comprising dodecanoic acid, tridecanoic acid and tetradecanoic acid.

14. The insulin derivative according to claim 1, wherein Xaa at position A21 is Ala, Gln, Gly or Ser.

15. The insulin derivative according to claim 1, wherein Xaa at position B3 is Asp, Gln or Thr.

16. The insulin derivative according to claim 1, wherein Xaa at position B1 is deleted.

17. A pharmaceutical composition for the treatment of diabetes in a patient in need of such treatment, comprising a therapeutically effective amount of an insulin derivative according to claim 1 together with a pharmaceutically acceptable carrier.

18. A pharmaceutical composition for the treatment of diabetes in a patient in need of such treatment, comprising a therapeutically effective amount of an insulin derivative according to claim 1, in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

19. A method of treating diabetes in a patient in need of such a treatment, comprising administering to the patient a therapeutically effective amount of an insulin derivative according to claim 1 together with a pharmaceutically acceptable carrier.

20. A method of treating diabetes in a patient in need of such a treatment, comprising administering to the patient a therapeutically effective amount of an insulin derivative according to claim 1 in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

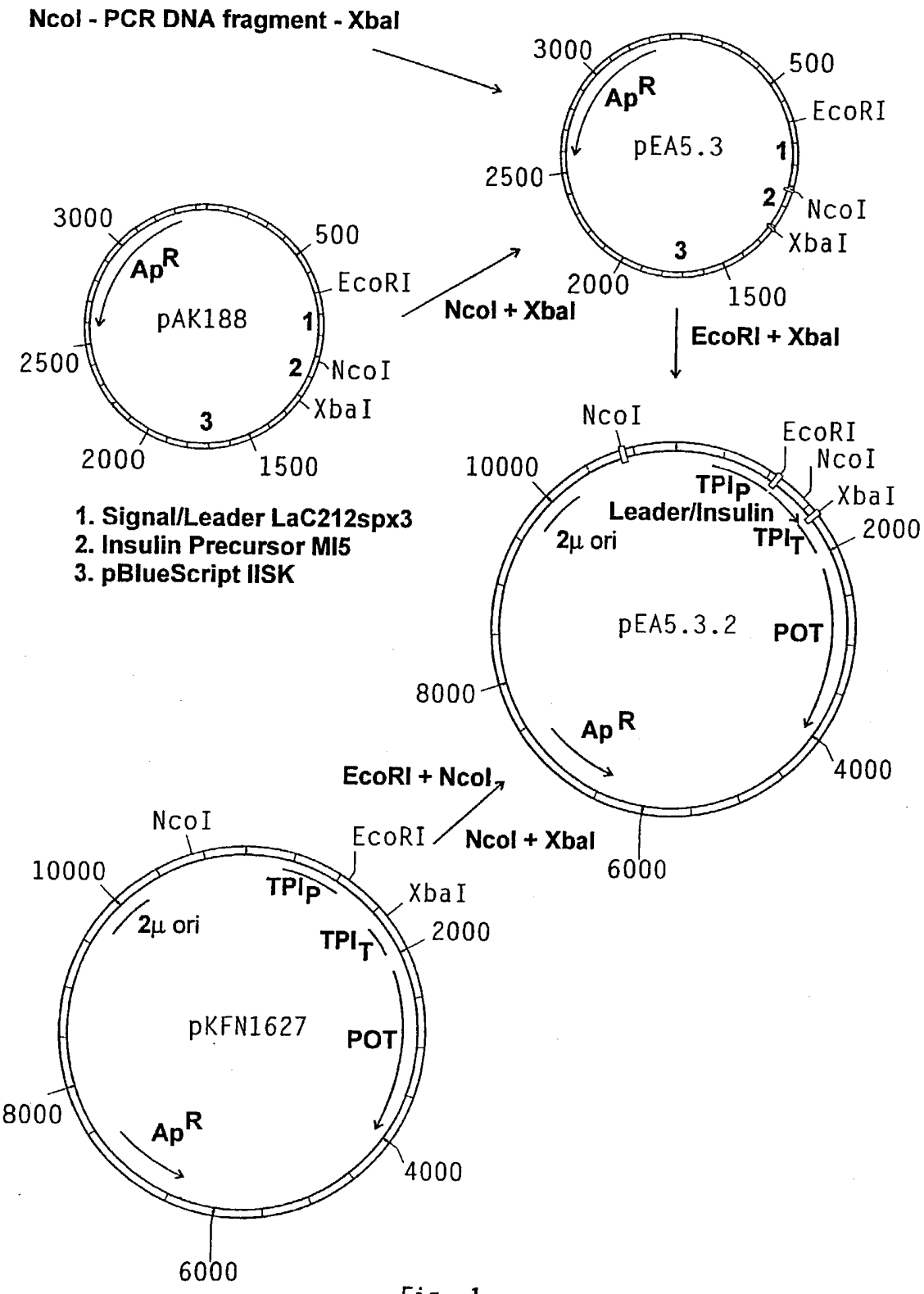

NcoI - PCR DNA fragment - XbaI
3000
500
ApR
EcoRI
pEA5.3
1
2500
2
NcoI
XbaI
3
2000
1500
NcoI + XbaI
EcoRI + XbaI
3000
500
ApR
EcoRI
pAK188
1
2500
2
NcoI
XbaI
3
2000
1500
1. Signal/Leader LaC212spx3
2. Insulin Precursor MI5
3. pBlueScript IISK
NcoI
EcoRI
NcoI
10000
TPIP
Leader/Insulin
XbaI
2µ ori
TPIT
2000
pEA5.3.2
POT
8000
ApR
4000
6000
EcoRI + NcoI
NcoI + XbaI
NcoI
EcoRI
10000
TPIP
XbaI
2µ ori
TPIT
2000
pKFN1627
POT
8000
ApR
4000
6000

Fig. 1

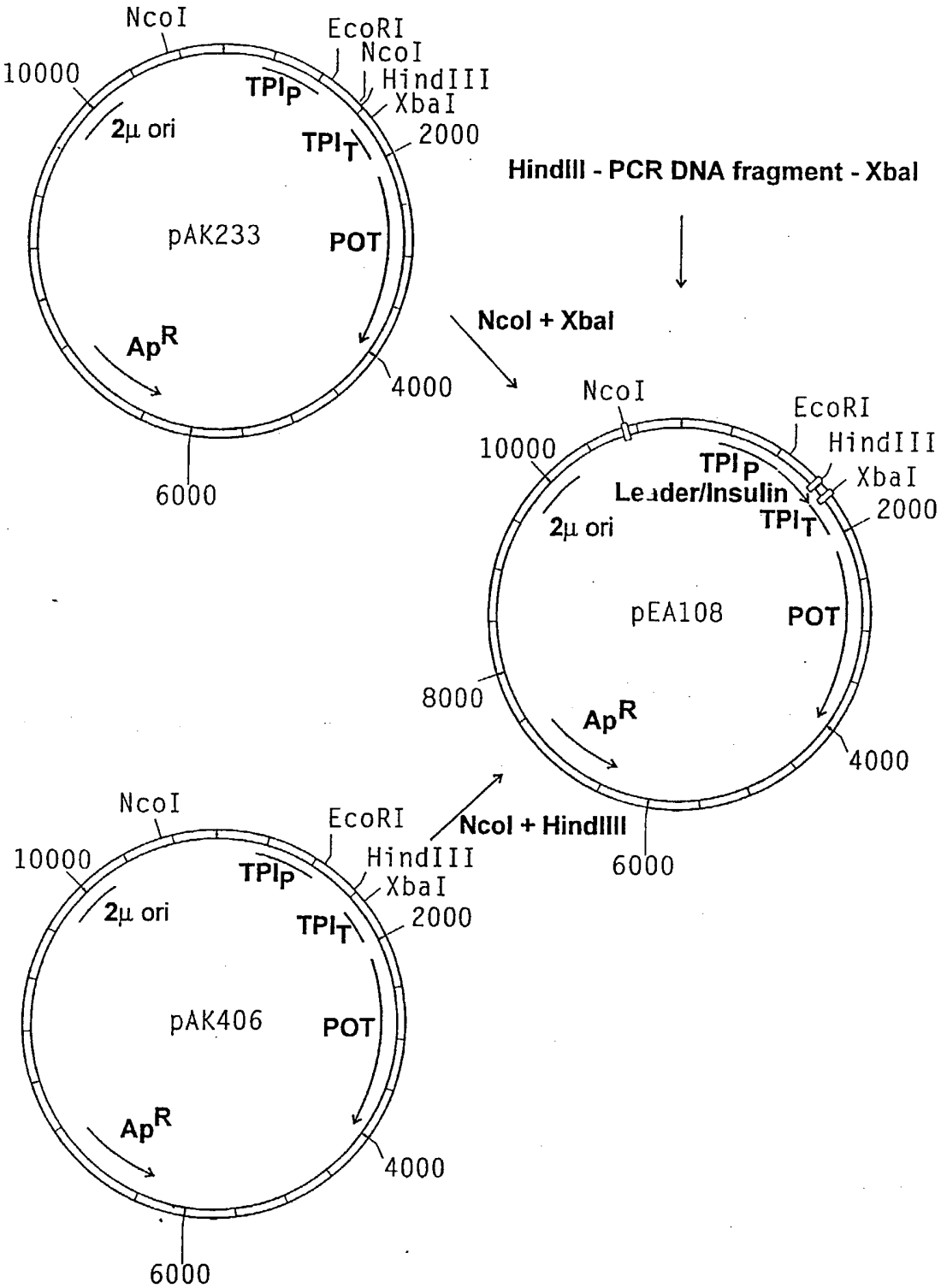

NcoI
EcoRI
NcoI
HindIII
XbaI
10000
TPIP
2μ ori
TPIT
2000
pAK233
POT
ApR
4000
6000
HindIII - PCR DNA fragment - XbaI
NcoI + XbaI
NcoI
EcoRI
HindIII
XbaI
10000
TPIP
Leader/Insulin
2μ ori
TPIT
2000
pEA108
POT
8000
ApR
4000
6000
NcoI + HindIII
NcoI
EcoRI
HindIII
XbaI
10000
TPIP
2μ ori
TPIT
2000
pAK406
POT
ApR
4000
6000

Fig. 2

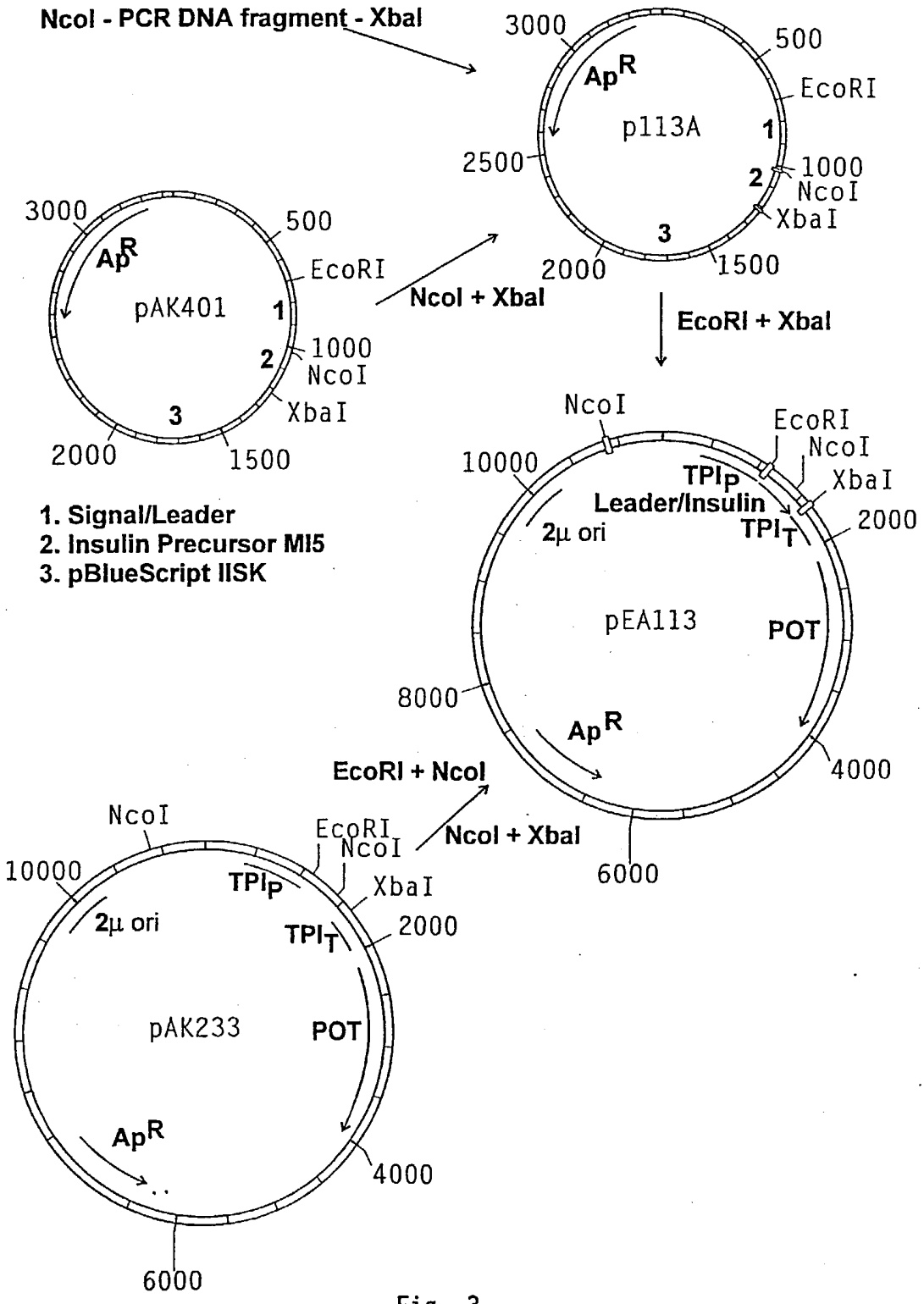

NcoI - PCR DNA fragment - XbaI
3000
500
EcoRI
ApR
p113A
1
2500
2
1000
NcoI
XbaI
3
2000
1500
3000
500
ApR
EcoRI
pAK401
1
2
1000
NcoI
3
XbaI
2000
1500
NcoI + XbaI
EcoRI + XbaI
1. Signal/Leader
2. Insulin Precursor MI5
3. pBlueScript IISK
NcoI
EcoRI
NcoI
10000
TPIP
XbaI
Leader/Insulin
2µ ori
TPIT
2000
pEA113
POT
8000
ApR
4000
6000
EcoRI + NcoI
NcoI + XbaI
NcoI
EcoRI
NcoI
10000
TPIP
XbaI
2µ ori
TPIT
2000
pAK233
POT
ApR
4000
6000

Fig. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 94/00347

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/62, A61K 38/28
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, WPI, CA, CLAIMS, JAPIO

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Patent Abstracts of Japan, Vol 14,No 7, C-673, abstract of JP, A, 1--254699 (KODAMA K.K.), 11 October 1989 (11.10.89) --	1-18
A	US, A, 3823125 (N. H. GRANT ET AL), 9 July 1974 (09.07.74) --	1-18
A	DE, B2, 2209835 (BAYER AG), 29 April 1976 (29.04.76) --	1-18
A	US, A, 3868356 (D. G. SMYTH), 25 February 1975 (25.02.75) --	1-18

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 December 1994	05 01-1995
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Elisabeth Carlborg Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 94/00347

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP, A2, 0127535 (HADASSAH MEDICAL ORGANIZATION), 5 December 1984 (05.12.84) -- --------	1-18

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 94/00347

Box I **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 19, 20
because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

26/11/94

International application No.
PCT/DK 94/00347

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United States Patent [19]

Norup et al.

[11] Patent Number: 5,866,538

[45] Date of Patent: Feb. 2, 1999

[54] **INSULIN PREPARATIONS CONTAINING NACL**

[75] Inventors: **Elsebeth Norup**, Jyllinge; **Liselotte Langkjær**, Klampenborg; **Svend Havelund**, Bagsvaerd, all of Denmark

[73] Assignee: **Novo Nordisk A/S**, Bagsvaerd, Denmark

[21] Appl. No.: **879,991**

[22] Filed: **Jun. 20, 1997**

Related U.S. Application Data

[60] Provisional application No. 60/020,927, Jun. 27, 1996.

[30] **Foreign Application Priority Data**

Jun. 20, 1996 [DK] Denmark 685/96

[51] **Int. Cl.**[6] **A61K 38/28**; C07K 14/62

[52] **U.S. Cl.** **514/3**; 530/389.2; 530/388.24; 530/303; 530/304

[58] **Field of Search** 514/3

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Primary Examiner—Cecilia J. Tsang
Assistant Examiner—Michael Borin
Attorney, Agent, or Firm—Steve T. Zelson, Esq.; Reza Green, Esq.; Carol E. Rozek, Esq.

[57] **ABSTRACT**

Insulin preparations of superior chemical stability, comprising human insulin or an analogue or derivative thereof, glycerol and/or mannitol, and 5 to 100 mM of a halogenide are disclosed.

17 Claims, No Drawings

INSULIN PREPARATIONS CONTAINING NACL

INTRODUCTION

This application is a continuation of provisional application Ser. No. 60/020,927, filed Jun. 27, 1996.

The present invention relates to aqueous insulin preparations comprising human insulin or an analogue or derivative thereof, which preparations have superior chemical stability. The invention furthermore relates to parenteral formulations comprising such insulin preparations and to a method for improving the chemical stability of insulin preparations.

BACKGROUND OF THE INVENTION

Diabetes is a general term for disorders in man having excessive urine excretion as in diabetes mellitus and diabetes insipidus. Diabetes mellitus is a metabolic disorder in which the ability to utilize glucose is more or less completely lost. About 2% of all people suffer from diabetes.

Since the introduction of insulin in the 1920's, continuos strides have been made to improve the treatment of diabetes mellitus. To help avoid extreme glycemia levels, diabetic patients often practice multiple injection therapy, whereby insulin is administered with each meal.

In the treatment of diabetes mellitus, many varieties of insulin preparations have been suggested and used, such as regular insulin, Semilente® insulin, isophane insulin, insulin zinc suspensions, protamine zinc insulin, and Ultralente® insulin. As diabetic patients are treated with insulin for several decades, there is a major need for safe and life quality improving insulin preparations. Some of the commercial available insulin preparations are characterized by a fast onset of action and other preparations have a relatively slow onset but show a more or less prolonged action. Fast acting insulin preparations are usually solutions of insulin, while retarded acting insulin preparations can be suspensions containing insulin in crystalline and/or amorphous form precipitated by addition of zinc salts alone or by addition of protamine or by a combination of both. In addition, some patients are using preparations having both a fast onset of action and a more prolonged action. Such a preparation may be an insulin solution wherein protamine insulin crystals are suspended. Some patients do themselves prepare the final preparation by mixing an insulin solution with a suspension preparation in the ratio desired by the patient in question.

Human insulin consists of two polypeptide chains, the so-called A and B chains which contain 21 and 30 amino acids, respectively. The A and B chains are interconnected by two cystine disulphide bridges. Insulin from most other species has a similar construction, but may not contain the same amino acids at the positions corresponding in the chains as in human insulin.

The development of the process known as genetic engineering has made it possible easily to prepare a great variety of insulin compounds being analogous to human insulin. In these insulin analogues, one or more of the amino acids have been substituted with other amino acids which can be coded for by the nucleotide sequences. As human insulin, as explained above, contains 51 amino acid residues, it is obvious that a large number of insulin analogues is possible and, in fact, a great variety of analogues with interesting properties have been prepared. In human insulin solutions with a concentration of interest for injection preparations, the insulin molecule is present in associated form as a

hexamer (Brange et al. Diabetes Care 13, (1990), 923–954). After subcutaneous injection, it is believed that the rate of absorption by the blood stream is dependent of the size of the molecule, and it has been found that insulin analogues with amino acid substitutions which counteract or inhibit this hexamer formation have an unusual fast onset of action (Brange et al.: Ibid). This is of great therapeutic value for the diabetic patient.

Pharmaceutical preparations which are based on analogues of human insulin have e.g. been presented by Heinemann et al., Lutterman et al. and Wiefels et al. at the "Frontiers in Insulin Pharmacology" International Symposium in Hamburg, 1992.

Furthermore, U.S. Pat. No. 5,474,978 discloses a rapid acting parenteral formulation comprising a human insulin analogue hexamer complex consisting of six monomeric insulin analogues, zinc ions and at least three molecules of a phenolic derivative.

Normally, insulin preparations are administered by subcutaneous injection. What is important for the patient, is the action profile of the insulin preparation which is the action of insulin on the glucose metabolism as a function of the time from the injection. In this profile, inter alia, the time for the onset, the maximum value and the total duration of action are important. A variety of insulin preparations with different action profiles are desired and requested by the patients. One patient may, on the same day, use insulin preparations with very different action profiles. The action profile requested is, for example, depending on the time of the day and the amount and composition of any meal eaten by the patient.

Equally important for the patient is the chemical stability of the insulin preparations, especially due to the abundant use of pen-like injection devices such as devices which contain Penfill® cartridges, in which an insulin preparation is stored until the entire cartridge is empty. This may last for at least 1 to 2 weeks for devices containing 1.5–3.0 ml cartridges. During storage, covalent chemical changes in the insulin structure occur. This may lead to formation of molecules which are less active and potentially immunogenic such as deamidation products and higher molecular weight transformation products (dimers, polymers, etc.). A comprehensive study on the chemical stability of insulin is given in by Jens Brange in "Stability of Insulin", Kluwer Academic Publishers, 1994.

Acta Pharmaceutica Nordica 4(4), 1992, pp. 149–158 discloses insulin preparations in which the sodium chloride concentration has been varied in the range of 0 to 250 mM. However, the major part of the preparations, including all preparations which additionally comprises glycerol, contains a rather high amount of sodium chloride, i.e. 0.7% corresponding approximately to a concentration of 120 mM. It is stated in this document that whereas sodium chloride generally has a stabilizing effect on insulin preparations, glycerol and glucose lead to increased chemical deterioration.

Surprisingly, however, it has now been shown that insulin preparations of superior chemical stability can be obtained in the presence of glycerol and/or mannitol and rather low halogenide concentrations.

DESCRIPTION OF THE INVENTION

By "analogue of human insulin" as used herein is meant human insulin in which one or more amino acids have been deleted and/or replaced by other amino acids, including non-codeable amino acids, or human insulin comprising additional amino acids, i.e. more than 51 amino acids.

By "derivative of human insulin" as used herein is meant human insulin or an analogue thereof in which at least one organic substituent is bound to one or more of the amino acids.

In the present context the unit "U" corresponds to 6 nmol.

The present invention relates to an aqueous insulin preparation comprising:

human insulin, an analogue thereof and/or a derivative thereof,

glycerol and/or mannitol, and

5 to 100 mM of a halogenide.

The above insulin preparation has a high chemical stability which e.g. is reflected in a reduction in the formation of dimers and polymers and desamido insulins after storage. Furthermore, the physical stability is not deteriorated by the presence of the rather low amount of halogenide, and the insulin does not precipitate by long-term storage of the insulin preparations.

The halogenide is preferably an alkali or alkaline earth halogenide, more preferably a chloride such as sodium chloride.

Glycerol and/or mannitol is preferably present in an amount corresponding to a concentration of 100 to 250 mM, more preferably 140 to 250 mM, even more preferably 160 to 200 mM.

The present invention is particularly advantageous in connection with preparations comprising analogues and/or derivatives of human insulin. Thus, the insulin preparation according to the invention preferably comprises one or more fast-acting analogues of human insulin, in particular analogues wherein position B28 is Asp, Lys, Leu, Val or Ala and position B29 is Lys or Pro; or des(B28–B30), des(B27) or des(B30) human insulin. The insulin analogue is preferably selected from analogues of human insulin wherein position B28 is Asp or Lys, and position B29 is Lys or Pro. The most preferred analogues are Asp^{B28} human insulin or $Lys^{B28}Pro^{B29}$ human insulin.

In this embodiment, the insulin preparation preferably comprises 5 to 60 mM, more preferably 5 to 40 mM, of a halogenide.

In another embodiment the insulin preparation according to the invention comprises an insulin derivative having a protracted profile of action such as insulins having one or more lipophilic substituents. The preferred lipophilic insulins are acylated insulins, including those described in WO 95/07931 (Novo Nordisk A/S), e.g. human insulin derivatives wherein the ε-amino group of Lys^{B29} contains an acyl substituent which comprises at least 6 carbon atoms.

The preferred insulins derivatives are the following:

B29-N$^{\varepsilon}$-myristoyl-des(B30) human insulin, B29-N$^{\varepsilon}$-palmitoyl-des(B30) human insulin, B29-N$^{\varepsilon}$-myristoyl human insulin, B29-N$^{\varepsilon}$-palmitoyl human insulin, B28-N$^{\varepsilon}$-myristoyl Lys^{B28} Pro^{B29} human insulin, B28-N$^{\varepsilon}$-palmitoyl Lys^{B28} Pro^{B29} human insulin, B30-N$^{\varepsilon}$-myristoyl-$Thr^{B29}Lys^{B30}$ human insulin, B30-N$^{\varepsilon}$-palmitoyl-$Thr^{B29}Lys^{B30}$ human insulin, B29-N$^{\varepsilon}$-(N-palmitoyl-γ-glutamyl)-des(B30) human insulin, B29-N$^{\varepsilon}$-(N-lithocholyl-γ-glutamyl)-des(B30) human insulin and B29-N$^{\varepsilon}$-(ω-carboxyheptadecanoyl)-des(B30) human insulin, B29-N$^{\varepsilon}$-(ω-carboxyheptadecanoyl) human insulin; the most preferred being B29-N$^{\varepsilon}$-myristoyl-des(B30) human insulin.

In this embodiment, the insulin preparation preferably comprises 10 to 100 mM, more preferably 10 to 70 mM, of a halogenide.

In a particular embodiment, the insulin preparation of the invention comprises an insulin analogue as well as an insulin derivative.

In a preferred embodiment of the invention the insulin preparation comprises:

60 to 3000 nmol/ml, preferably 240 to 1200 nmol/ml, of human insulin or insulin analogue or derivative,

10 to 40 μg Zn/100 U insulin, preferably 10 to 26 μg Zn/100 U insulin, and

0 to 5 mg/ml, preferably 0 to 4 mg/ml, of a phenolic compound.

As a phenolic compound, 0.5 to 4.0 mg/ml, preferably 0.6 to 4.0 mg/ml, of m-cresol or 0.5 to 4.0 mg/ml, preferably 1.4 to 4.0 mg/ml, of phenol, or a mixture thereof, is advantageously employed.

The insulin preparation of the present invention may furthermore contain other ingredients common to insulin preparations, for example zinc complexing agents such as citrate, and phosphate buffers.

The present invention furthermore relates to a parenteral pharmaceutical formulation comprising an insulin preparation of the invention.

Moreover, the present invention is concerned with a method for improving the chemical stability of an insulin preparation comprising human insulin or an analogue or a derivative thereof, which method comprises adding glycerol and/or mannitol and 5 to 100 mM of a halogenide to said preparation.

The invention is further illustrated by the following examples which, however, are not to be construed as limiting.

EXAMPLE I

Solutions containing 100 U/ml Asp^{B28} human insulin, 2.6 mg/ml phenol, 16 mg/ml glycerol and varying amounts of Zn and sodium chloride were prepared. The pH was varied in the range of 7.2 to 7.5. Stability data after 4 weeks at 37° C. are presented in the following Table 1.

TABLE 1

μg Zn/100 U insulin	NaCl (mM)	pH	Asp^{B28} Desamido insulins formed (%)	Di- & polymers formed (%)
13.1	0	7.2	3.44	1.35
		7.5	3.57	1.36
	5	7.2	3.48	1.53
		7.5	3.31	1.49
	20	7.2	2.54	1.72
		7.5	2.47	1.26
16.3	0	7.2	3.35	1.44
		7.4	3.41	1.46
	5	7.2	1.74	0.95
		7.5	2.58	1.38
	20	7.2	1.91	1.05
		7.5	2.00	1.31
19.6	0	7.2	3.07	1.57
		7.5	2.85	1.80
	5	7.2	2.71	1.36
		7.5	2.24	1.46
	20	7.2	1.56	1.15
		7.5	1.68	1.13
22.8	0	7.2	2.71	2.52
		7.5	2.34	1.45
	5	7.2	2.18	1.95
		7.5	1.90	1.19
	20	7.2	1.51	1.05
		7.5	1.46	1.09

EXAMPLE II

Insulin preparations containing dissolved Asp^{B28} human insulin with varying concentrations of sodium chloride was prepared in the following way:

370.4 mg AspB28 human insulin was dissolved in water by adding 1.6 ml 0.2N HCl and 49 μl zinc chloride solution (40 mg Zn/ml). 40 g of a solution containing 40 mg/ml glycerol, 3.75 mg/g phenol and 4.30 mg/g m-cresol was added to the insulin solution while mixing. 20 g of a solution containing a) 12.0 mg/g disodium phosphate dihydrate+5 μl/g 2N sodium hydroxide, b) 12.0 mg/g disodium phosphate dihydrate+5 μl/g 2N sodium hydroxide+5 mg/g sodium chloride or c) 12.0 mg/g disodium phosphate dihydrate+5 μl/g 2N sodium hydroxide+10 mg/g sodium chloride was added while mixing. pH was adjusted to pH 7.40±0.05 and water added up to 100 ml. The Asp^{B28} Human insulin preparations were introduced into Penfill® cartridges and subjected to stability tests at 25° C. and 37° C. The stability data obtained at the two different temperatures and at a phosphate concentration of 13.5 mM, 19.6 μg Zn/100 U insulin and pH=7.4 are summarized in Table 2.

TABLE 2

NaCl added (mM)	Total conc. Of Cl^- (mM)	Asp^{B28} Des-amido insulins formed (%)	Di- & polymers formed (%)
	Data after 8 weeks at 37° C.		
0	4.4	7.0	1.86
17	20.8	4.2	1.29
34	37.8	3.5	1.07
	Data after 8 months at 25° C.		
0	4.4	6.4	1.0
17	20.8	4.1	0.8
34	37.8	3.7	0.8

EXAMPLE III

Insulin preparations containing dissolved Asp^{B28} human insulin with varying concentrations of sodium chloride was prepared in the following way:

369.4 mg Asp^{B28} human insulin was dissolved in water by adding 1.6 ml 0.2N HCl and 49 μl zinc chloride solution (40 mg Zn/ml). 40 g of a solution containing 40 mg/g glycerol, 3.75 mg/g phenol and 4.30 mg/g m-cresol was added to the solution while mixing. 10 g of a solution containing 24.0 mg/g disodium phosphate dihydrate and 11 μl/g 2N sodium hydroxide was added while mixing. Finally varying amounts (0 g to 4.38 g) of a solution containing 40 mg/g sodium chloride were added while mixing up to a sodium chloride concentration mentioned in Table 4. pH was adjusted to 7.40±0.05 and water added up to 100 ml. The Asp^{B28} Human insulin preparations were introduced into Penfill® cartridges and subjected to stability tests at 25° C. and 37° C. The stability data obtained at the two different temperatures and at a phosphate concentration of 13.5 mM are summarized in Table 3.

TABLE 3

NaCl added (mM)	Total conc. of Cl^- (mM)	Asp^{B28} Des-amido insulins formed (%)	Di- & polymers formed (%)
	Stability data after 6 weeks at 37° C.		
5	8.5	4.1	0.99
12.5	16.3	3.6	0.92
20	23.8	3.0	0.87
25	28.8	3.0	0.82
30	33.8	2.8	0.80

TABLE 3-continued

NaCl added (mM)	Total conc. of Cl^- (mM)	Asp^{B28} Des-amido insulins formed (%)	Di- & polymers formed (%)
	Stability data after 12 weeks at 25° C.		
0	3.8	2.7	0.36
5	8.5	2.3	0.32
12.5	16.3	1.8	0.39
20	23.8	1.7	0.39
25	28.8	1.8	0.38
30	33.8	1.7	0.38

EXAMPLE IV

Insulin preparations containing dissolved Asp^{B28} human insulin with varying concentrations of phosphate and sodium chloride was prepared in the following way:

375.7 mg Asp^{B28} human insulin was dissolved in water by adding 1.6 ml 0.2N HCl and 49 μl zinc chloride solution (40 mg Zn/ml). 20 g of a solution containing 80 mg/g glycerol, 7.50 mg/g phenol and 8.60 mg/g m-cresol was added to the solution while mixing. Varying amounts (3.71 g to 6.71 g) of a solution containing 24.0 mg/g disodium phosphate dihydrate and 11 μl/g 2N sodium hydroxide was added while mixing, finally varying amounts (0 g to 3.65 g) of a solution containing 40 mg/g sodium chloride were added while mixing so as to obtain a sodium chloride concentration mentioned in table 6. pH was adjusted to pH 7.40±0.05 and water added up to 100 ml. The Asp^{B28} Human insulin preparations were introduced into Penfill® cartridges and subjected to stability tests at 25° C. and 37° C. The stability data at the two different temperatures and three different phosphate concentrations and at 19.6 μg Zn/100 U insulin and pH=7.4 are summarized in Tables 4, 5 and 6.

TABLE 4

NaCl added (mM)	Total conc. of Cl^- (mM)	Phosphate conc. (mM)	Asp^{B28} Des-amido insulins formed (%)	Di- & polymers formed (%)
		Data after 6 weeks at 37° C.		
0	3.8	5	4.7	1.4
5	8.8	5	3.7	1.3
10	13.8	5	3.4	1.2
15	18.8	5	3.1	1.1
20	23.8	5	2.7	1.1
25	28.8	5	3.0	0.9
		Data after 12 weeks at 25° C.		
0	3.8	5	2.2	0.5
5	8.8	5	1.7	0.4
10	13.8	5	1.5	0.4
15	18.8	5	1.4	0.4
20	23.8	5	1.3	0.4
25	28.8	5	1.3	0.4

TABLE 5

NaCl added (mM)	Total conc. of Cl^- (mM)	Phosphate conc. (mM)	Asp^{B28} Des-amido insulins formed (%)	Di- & polymers formed (%)
		Data after 6 weeks at 37° C.		
0	3.8	7	4.3	1.2
5	8.8	7	3.6	1.2

TABLE 5-continued

NaCl added (mM)	Total conc. of Cl^- (mM)	Phosphate conc. (mM)	Asp^{B28} Des-amido insulins formed (%)	Di- & polymers formed (%)
10	13.8	7	3.1	1.1
15	18.8	7	3.1	1.0
20	23.8	7	2.9	1.0
25	28.8	7	2.8	1.1
		Data after 12 weeks at 25° C.		
0	3.8	7	2.0	0.5
5	8.8	7	1.7	0.4
10	13.8	7	1.4	0.4
15	18.8	7	1.5	0.4
20	23.8	7	1.4	0.4
25	28.8	7	1.3	0.4

TABLE 6

NaCl added (mM)	Total conc. of Cl^- (mM)	Phosphate conc. (mM)	Asp^{B28} Des-amido insulins formed (%)	Di- & polymers formed (%)
		Data after 6 weeks at 37° C.		
0	3.8	9	4.9	1.2
5	8.8	9	4.0	1.1
10	13.8	9	3.7	1.0
15	18.8	9	3.5	1.0
20	23.8	9	3.5	1.0
25	28.8	9	3.1	0.9
		Data after 12 weeks at 25° C.		
0	3.8	9	n.d.	0.4
5	8.8	9	1.8	0.4
10	13.8	9	1.5	0.4
15	18.8	9	1.5	0.4
20	23.8	9	1.6	0.4
25	28.8	9	1.4	0.4

EXAMPLE V

Solutions containing 0.6 mM B29-N^{ϵ}-myristoyl-des(B30) human insulin, 1.5 or 4.0 mg/ml phenol, 5 mM sodium phosphate, 13.1 μg/ml Zn, and varying amounts of sodium chloride and mannitol were prepared. pH was adjusted to 7.4. Stability data (formation of dimers and polymers) after storage at 25° C. for 13 weeks or 37° C. for 8 weeks are presented in the following table 7.

TABLE 7

NaCl (mM)	Mannitol (mg/ml)	Phenol 1.5 mg/ml	Phenol 4.0 mg/ml
		Di- & polymers (%) formed after 8 weeks at 37° C.	
20	31	0.77	0.77
50	22	0.71	0.71
75	13	0.65	0.70
100	5	0.66	0.68
		Di- & polymers (%) formed after 13 weeks at 25° C.	
20	31	0.40	0.42
50	22	0.35	0.37
75	13	0.34	0.39
100	5	0.31	0.37

EXAMPLE VI

Solutions containing 0.6 mM B29-N^{ϵ}-myristoyl des(B30) human insulin, 1.5 mg/ml phenol and 1.72 mg/ml m-cresol, 16 mg/ml glycerol or 36 mg/ml mannitol, 13.1 μg/ml Zn, 7 mM sodium phosphate and varying amounts of sodium chloride were prepared. pH was adjusted to 7.5. Stability data (formation of dimers and polymers) after storage at 25° C. for 13 weeks or 37° C. for 8 weeks are presented in the following table 8.

TABLE 8

NaCl (mM)	Glycerol 16 mg/ml	Mannitol 36 mg/ml
	Di- & polymers (%) formed after 8 weeks at 37° C.	
5	2.55	2.28
10	2.25	1.90
20	1.82	1.61
30	1.83	n.d.
40	1.78	1.56
50	1.68	n.d.
	Di- & polymers (%) formed after 13 weeks at 25° C.	
5	1.08	1.05
10	0.98	0.84
20	0.80	0.71
30	0.80	n.d.
40	0.79	0.70
50	0.72	n.d.

We claim:

1. A pharmaceutical formulation comprising:

a polypeptide selected from the group consisting of human insulin, an analogue thereof, a derivative thereof, and combinations of any of the foregoing;

glycerol, mannitol, or glycerol and mannitol; and

5 to 100 mM of a halogenide.

2. A pharmaceutical formulation according to claim 1, wherein the halogenide is an alkali or alkaline earth halogenide.

3. A pharmaceutical formulation according to claim 1, wherein said glycerol or mannitol is present at a concentration of 100 to 250 mM.

4. A pharmaceutical formulation according to claim 1, wherein said polypeptide is an analogue of human insulin selected from the group consisting of: (i) an analogue wherein position B28 is Asp, Lys, Leu, Val or Ala and position B29 is Lys or Pro; and (ii) des(B28–B30), des(B27) or des(B30) human insulin.

5. A pharmaceutical formulation according to claim 4, wherein said polypeptide is an analogue of human insulin wherein position B28 is Asp or Lys, and position B29 is Lys or Pro.

6. A pharmaceutical formulation according to claim 4, wherein said polypeptide is des(B30) human insulin.

7. A pharmaceutical formulation according to claim 1, wherein said halogenide is present at a concentration of 5 to 60 mM.

8. A pharmaceutical formulation according to claim 1, wherein said polypeptide is a derivative of human insulin having one or more lipophilic substituents.

9. A pharmaceutical formulation according to claim 8, wherein the insulin derivative is selected from the group consisting of B29-N^{ϵ}-myristoyl-des(B30) human insulin, B29-N^{ϵ}-palmitoyl-des(B30) human insulin, B29-N^{ϵ}-myristoyl human insulin, B29-N^{ϵ}-palmitoyl human insulin, B28-N^{ϵ}-myristoyl $Lys^{B28}Pro^{B29}$ human insulin, B28-N^{ϵ}-palmitoyl-$Lys^{B28}Pro^{B29}$ human insulin, B30-N^{ϵ}-myristoyl-$Thr^{B29}Lys^{B30}$ human insulin, B30-N^{ϵ}-palmitoyl-$Thr^{B29}Lys^{B30}$ human insulin, B29-N^{ϵ}-(N-palmitoyl-γ-glutamyl)-des(B30) human insulin, B29-N^{ϵ}(N-lithocholyl-

γ-glutamyl)-des(B30) human insulin, B29-N^{ϵ}-(ω-carboxyheptadecanoyl)-des(B30) human insulin and B29-N^{ϵ}-(ω-carboxyheptadecanoyl) human insulin.

10. A pharmaceutical formulation according to claim **9**, wherein the insulin derivative is B29-N^{ϵ}-myristoyl-des (B30) human insulin.

11. A pharmaceutical formulation according to claim **8**, wherein said halogenide is present at a concentration of 10 to 100 mM.

12. A pharmaceutical formulation according to claim **1**, comprising an insulin analogue as well as an insulin derivative.

13. A pharmaceutical formulation according to claim **1**, wherein said polypeptide is present at a concentration of 60 to 3000 nmol/ml.

14. A pharmaceutical formulation according to claim **1**, further comprising:

10 to 40 ug Zn/100 U insulin.

15. A pharmaceutical formulation according to claim **1**, further comprising:

0 to 5 mg/ml of a phenolic compound.

16. A pharmaceutical formulation according to claim **15**, comprising:

0.5 to 4.0 mg/ml of m-cresol or 0.5 to 4.0 mg/ml of phenol, or a mixture thereof.

17. A pharmaceutical formulation according to claim **1**, wherein the halogenide is sodium chloride.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,866,538
DATED : February 2, 1999
INVENTOR(S) : Norup et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Col. 1, line 22: delete "continuos" and insert --continuous--

Signed and Scaled this

Fifteenth Day of June, 1999

Attest:

Q. TODD DICKINSON

Attesting Officer

Acting Commissioner of Patents and Trademarks

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United States Patent [19]

Havelund et al.

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[54] **ACYLATED INSULIN**

[75] Inventors: **Svend Havelund**, Bagsvaerd; **John Halstrom**, Hundested; **Ib Jonassen**, Valby; **Asser Sloth Andersen**, Frederiksberg; **Jan Markussen**, Herlev, all of Denmark

[73] Assignee: **Novo Nordisk A/S**, Bagsvaerd, Denmark

[*] Notice: This patent is subject to a terminal disclaimer.

[21] Appl. No.: **08/975,365**

[22] Filed: **Nov. 20, 1997**

Related U.S. Application Data

[62] Continuation-in-part of application No. 08/400,256, Mar. 8, 1995, Pat. No. 5,750,497, which is a continuation-in-part of application No. 08/190,829, filed as application No. PCT/DK94/00347, Sep. 16, 1994, abandoned.

[30] **Foreign Application Priority Data**

Sep. 17, 1993 [DK] Denmark 104493

[51] **Int. Cl.**[7] **C07K 14/62**; A61K 38/28

[52] **U.S. Cl.** **514/3**; 514/866; 530/303; 530/304

[58] **Field of Search** 530/303.4; 514/24, 514/12, 866

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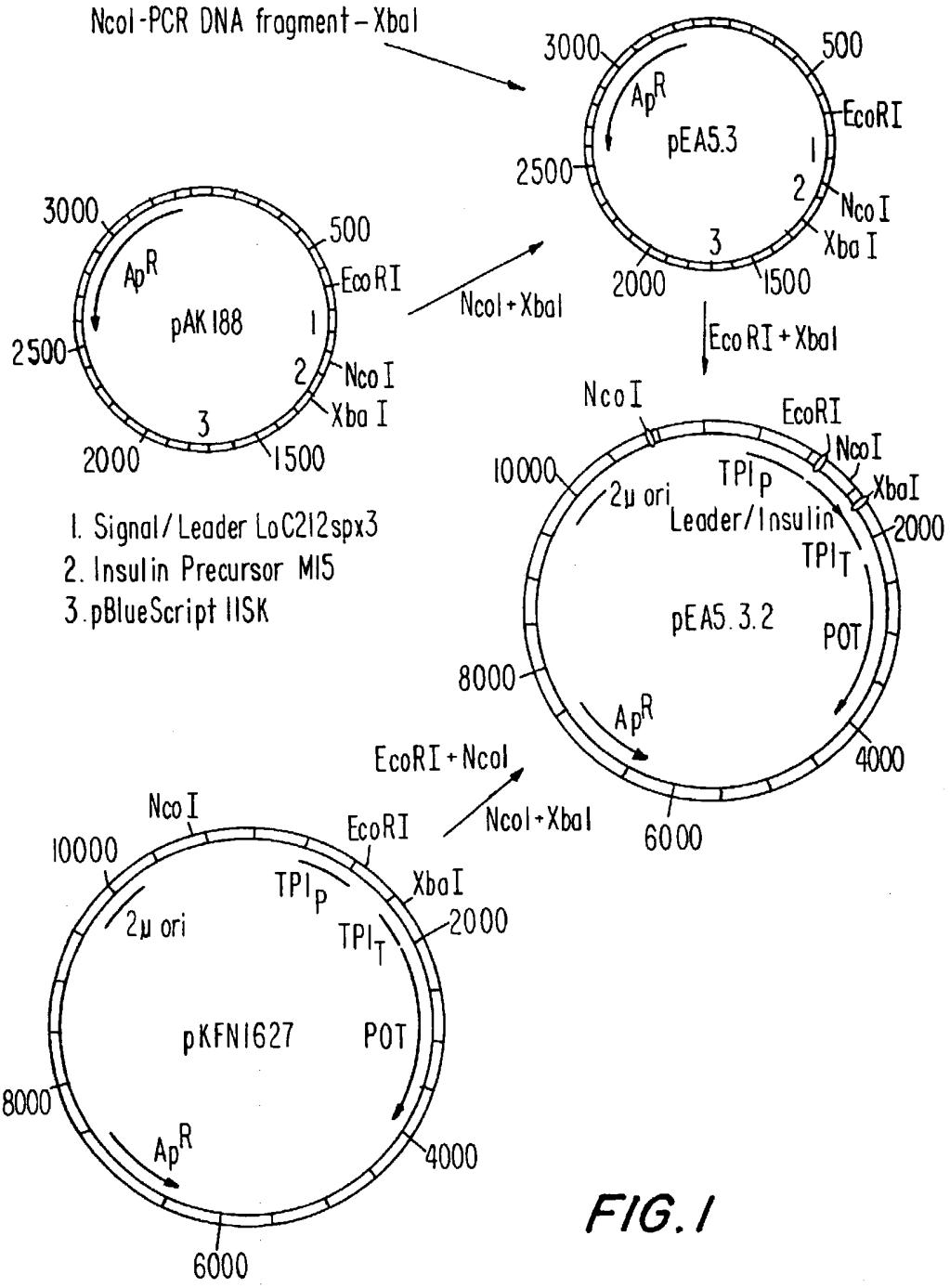
Primary Examiner—John Ulm
Assistant Examiner—Christine Saoud
Attorney, Agent, or Firm—Steve T. Zelson, Esq.; Elias Lambiris, Esq.

[57] **ABSTRACT**

The present invention relates to protracted human insulin derivatives in which the A21 and the B3 amino acid residues are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; Phe^{B1} may be deleted; the B30 amino acid residue is (a) a non-codable, lipophilic amino acid having from 10 to 24 carbon atoms, in which case an acyl group of a carboxylic acid with up to 5 carbon atoms is bound to the ϵ-amino group of Lys^{B29}; or (b) the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys, in any of which cases the ϵ-amino group of Lys^{B29} has a lipophilic substituent; and any Zn^{2+} complexes thereof with the proviso that when B30 is Thr or Ala and A21 and B3 are both Asn, and Phe^{B1} is present, then the insulin derivative is always present as a Zn^{2+} complex.

115 Claims, 3 Drawing Sheets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2
NcoI
XbaI
3
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1500
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EcoRI+XbaI
1. Signal/Leader LoC212spx3
2. Insulin Precursor MI5
3. pBlueScript IISK
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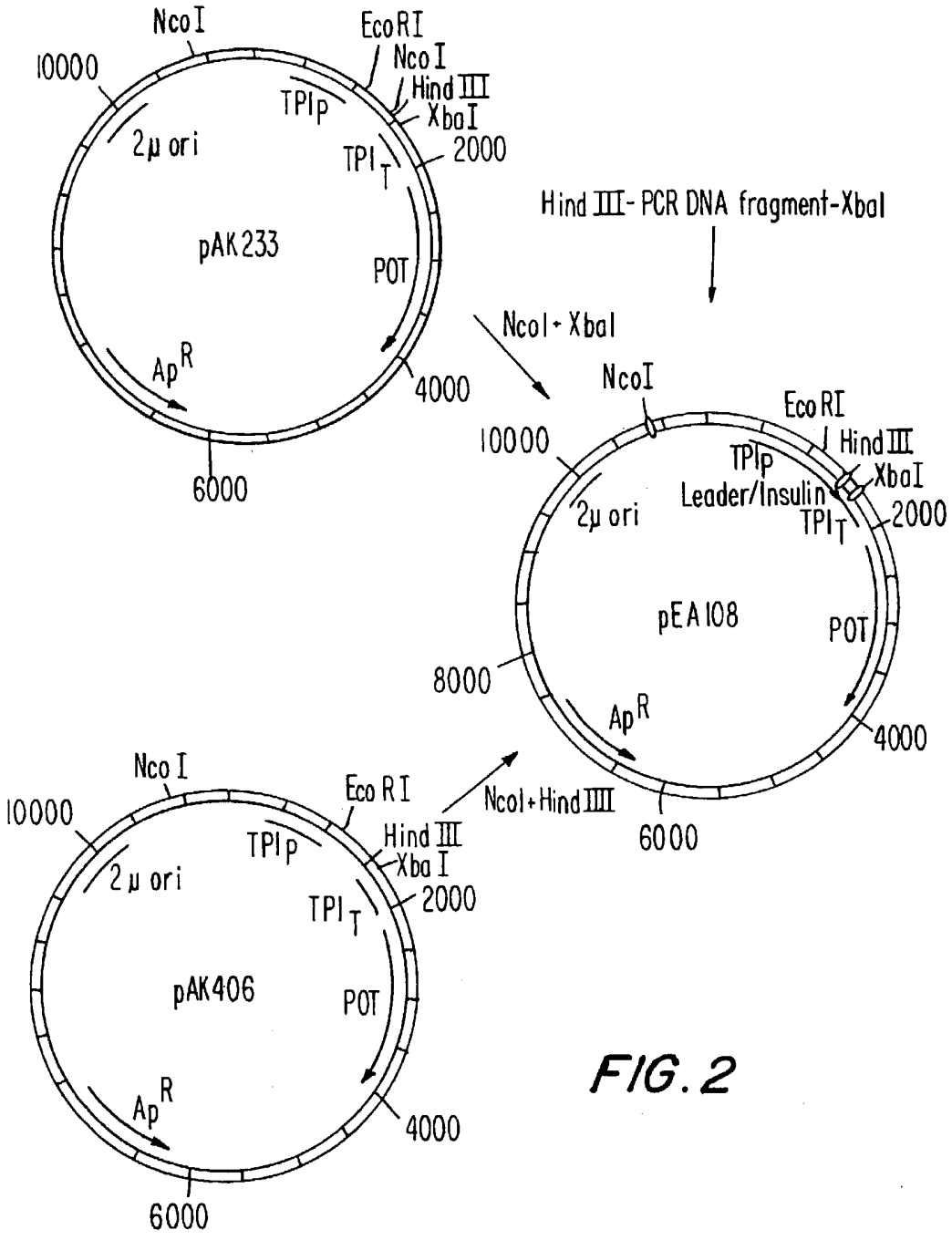
FIG. 1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NcoI + XbaI
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EcoRI
Hind III
XbaI
10000
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Leader/Insulin
2μ ori
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4000
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NcoI + Hind III
Nco I
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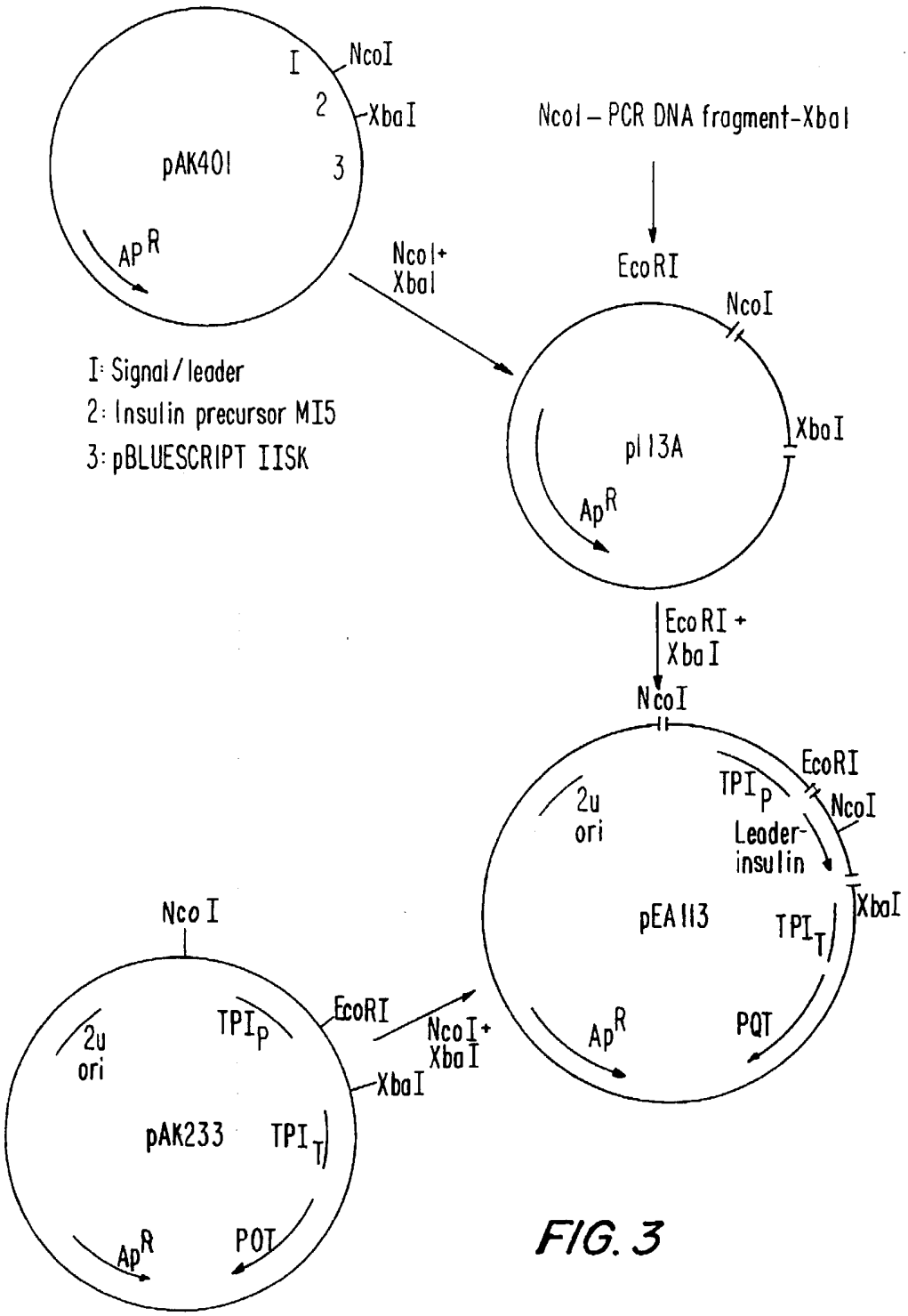
FIG. 2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NcoI
2
XbaI
pAK401
3
AP R
1: Signal / leader
2: Insulin precursor MI5
3: pBLUESCRIPT IISK
NcoI + XbaI
NcoI – PCR DNA fragment-XbaI
EcoRI
NcoI
XbaI
pI13A
Ap R
EcoRI + XbaI
NcoI
EcoRI
TPI P
NcoI
2u ori
Leader-insulin
XbaI
pEA113
TPI T
Ap R
POT
NcoI
EcoRI
NcoI + XbaI
2u ori
TPI P
XbaI
pAK233
TPI T
Ap R
POT

FIG. 3

ACYLATED INSULIN

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application Ser. No. 08/400,256 filed Mar. 8, 1995 now U.S. Pat. No. 5,750,997, which is a continuation-in-part of Ser No. 08/190,829 filed Feb. 2, 1994, now abandoned, and Ser. No. PCT/DK94/00347 filed Sep. 16, 1994, now abandoned, the contents of which are fully incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to novel human insulin derivatives which are soluble and have a protracted profile of action, to a method of providing such derivatives, to pharmaceutical compositions containing them, and to the use of such insulin derivatives in the treatment of diabetes.

BACKGROUND OF THE INVENTION

Many diabetic patients are treated with multiple daily insulin injections in a regimen comprising one or two daily injections of a protracted insulin to cover the basal requirement supplemented by bolus injections of a rapid acting insulin to cover the requirement related to meals.

Protracted insulin compositions are well known in the art. Thus, one main type of protracted insulin compositions comprises injectable aqueous suspensions of insulin crystals or amorphous insulin. In these compositions, the insulin compounds utilized typically are protamine insulin, zinc insulin or protamine zinc insulin.

Certain drawbacks are associated with the use of insulin suspensions. Thus, in order to secure an accurate dosing, the insulin particles must be suspended homogeneously by gentle shaking before a defined volume of the suspension is withdrawn from a vial or expelled from a cartridge. Also, for the storage of insulin suspensions, the temperature must be kept within more narrow limits than for insulin solutions in order to avoid lump formation or coagulation.

While it was earlier believed that protamines were non-immunogenic, it has now turned out that protamines can be immunogenic in man and that their use for medical purposes may lead to formation of antibodies (Samuel et al., Studies on the immunogenecity of protamines in humans and experimental animals by means of a micro-complement fixation test, Clin. Exp. Immunol. 33, pp. 252–260 (1978)).

Also, evidence has been found that the protamine-insulin complex is itself immunogenic (Kurtz et al., Circulating IgG antibody to protamine in patients treated with protamine-insulins. Diabetologica 25, pp. 322–324 (1983)). Therefore, with some patients the use of protracted insulin compositions containing protamines must be avoided.

Another type of protracted insulin compositions are solutions having a pH value below physiological pH from which the insulin will precipitate because of the rise in the pH value when the solution is injected. A drawback with these solutions is that the particle size distribution of the precipitate formed in the tissue on injection, and thus the timing of the medication, depends on the blood flow at the injection site and other parameters in a somewhat unpredictable manner. A further drawback is that the solid particles of the insulin may act as a local irritant causing inflammation of the tissue at the site of injection.

WO 91/12817 (Novo Nordisk A/S) discloses protracted, soluble insulin compositions comprising insulin complexes of cobalt(III). The protraction of these complexes is only intermediate and the bioavailability is reduced.

Human insulin has three primary amino groups: the N-terminal group of the A-chain and of the B-chain and the ε-amino group of Lys^{B29}. Several insulin derivatives which are substituted in one or more of these groups are known in the prior art. Thus, U.S. Pat. No. 3,528,960 (Eli Lilly) relates to N-carboxyaroyl insulins in which one, two or three primary amino groups of the insulin molecule has a carboxyaroyl group. No specifically $N^{\epsilon B29}$-substituted insulins are disclosed.

According to GB Patent No. 1.492.997 (Nat. Res. Dev. Corp.), it has been found that insulin with a carbamyl substitution at $N^{\epsilon B29}$ has an improved profile of hypoglycaemic effect.

JP laid-open patent application No. 1-254699 (Kodama Co., Ltd.) discloses insulin wherein a fatty acid is bound to the amino group of Phe^{B1} or to the ε-amino group of Lys^{B29} or to both of these. The stated purpose of the derivatisation is to obtain a pharmacologically acceptable, stable insulin preparation.

Insulins, which in the B30 position have an amino acid having at least five carbon atoms which cannot necessarily be coded for by a triplet of nucleotides, are described in JP laid-open patent application No. 57-067548 (Shionogi). The insulin analogues are claimed to be useful in the treatment of diabetes mellitus, particularly in patients who are insulin resistant due to generation of bovine or swine insulin antibodies.

By "insulin derivative" as used herein is meant a compound having a molecular structure similar to that of human insulin including the disulfide bridges between Cys^{A7} and Cys^{B7} and between Cys^{A20} and Cys^{B19} and an internal disulfide bridge between Cys^{A6} and Cys^{A11}, and which have insulin activity.

However, there still is a need for protracted injectable insulin compositions which are solutions and contain insulins which stay in solution after injection and possess minimal inflammatory and immunogenic properties.

One object of the present invention is to provide human insulin derivatives, with a protracted profile of action, which are soluble at physiological pH values.

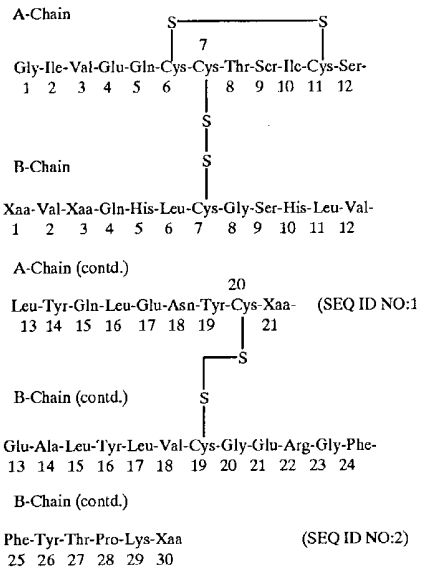
Another object of the present invention is to provide a pharmaceutical composition comprising the human insulin derivatives according to the invention.

It is a further object of the invention to provide a method of making the human insulin derivatives of the invention.

SUMMARY OF THE INVENTION

Surprisingly, it has turned out that certain human insulin derivatives, wherein the ε-amino group of Lys^{B29} has a lipophilic substituent, have a protracted profile of action and are soluble at physiological pH values.

Accordingly, in its broadest aspect, the present invention relates to an insulin derivative having the following sequence:



wherein

Xaa at positions A21 and B3 are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys;

Xaa at position B1 is Phe or is deleted;

Xaa at position B30 is (a) a non-codable, lipophilic amino acid having from 10 to 24 carbon atoms, in which case an acyl group of a carboxylic acid with up to 5 carbon atoms is bound to the ε-amino group of Lys^{B29}, (b) any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys, in which case the ε-amino group of Lys^{B29} has a lipophilic substituent or (c) deleted, in which case the ε-amino group of Lys^{B29} has a lipophilic substituent; and any Zn^{2+} complexes thereof, provided that when Xaa at position B30 is Thr or Ala, Xaa at positions A21 and B3 are both Asn, and Xaa at position B1 is Phe, then the insulin derivative is a Zn^{2+} complex.

In one preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; the A21 and the B3 amino acid residues are, independently, any amino acid residues which can be coded for by the genetic code except Lys, Arg and Cys; Phe^{B1} may be deleted; the ε-amino group of Lys^{B29} has a lipophilic substituent which comprises at least 6 carbon atoms; and 2–4 Zn^{2+} ions may be bound to each insulin hexamer with the proviso that when B30 is Thr or Ala and A21 and B3 are both Asn, and Phe^{B1} is not deleted, then 2–4 Zn^{2+} ions are bound to each hexamer of the insulin derivative.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; the A21 and the B3 amino acid residues are, independently, any amino acid residues which can be coded for by the genetic code except Lys, Arg and Cys, with the proviso that if the B30 amino acid residue is Ala or Thr, then at least one of the residues A21 and B3 is different from Asn; Phe^{B1} may be deleted; and the ε-amino group of Lys^{B29} has a lipophilic substituent which comprises at least 6 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; the A21 and the B3 amino acid residues are, independently, any amino acid residues which can be coded for by the genetic code except Lys, Arg and Cys; Phe^{B1} may be deleted; the ε-amino group of Lys^{B29} has a lipophilic substituent which comprises at least 6 carbon atoms; and 2–4 Zn^{2+} ions are bound to each insulin hexamer.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is Asp.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is Glu.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid residue is Thr.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is a lipophilic amino acid having at least 10 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is a lipophilic α-amino acid having from 10 to 24 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is a straight chain, saturated, aliphatic α-amino acid having from 10 to 24 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is D- or L-N^{ε}-dodecanoyllysine.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α-amino decanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α-amino undecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α-amino dodecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α-amino tridecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α-amino tetradecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α-amino pentadecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is α-amino hexadecanoic acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B30 amino acid is an α-amino acid.

In another preferred embodiment, the invention relates to a human insulin derivative in which the A21 amino acid residue is Ala.

In another preferred embodiment, the invention relates to a human insulin derivative in which the A21 amino acid residue is Gln.

In another preferred embodiment, the invention relates to a human insulin derivative in which the A21 amino acid residue is Gly.

In another preferred embodiment, the invention relates to a human insulin derivative in which the A21 amino acid residue is Ser.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B3 amino acid residue is Asp.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B3 amino acid residue is Gln.

In another preferred embodiment, the invention relates to a human insulin derivative in which the B3 amino acid residue is Thr.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ-amino group of Lys^{B29} has a lipophilic substituent which is an acyl group corresponding to a carboxylic acid having at least 6 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ-amino group of Lys^{B29} has a lipophilic substituent which is an acyl group, branched or unbranched, which corresponds to a carboxylic acid having a chain of carbon atoms 8 to 24 atoms long.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ-amino group of Lys^{B29} has a lipophilic substituent which is an acyl group corresponding to a fatty acid having at least 6 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ-amino group of Lys^{B29} has a lipophilic substituent which is an acyl group corresponding to a linear, saturated carboxylic acid having from 6 to 24 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ-amino group of Lys^{B29} has a lipophilic substituent which is an acyl group corresponding to a linear, saturated carboxylic acid having from 8 to 12 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ-amino group of Lys^{B29} has a lipophilic substituent which is an acyl group corresponding to a linear, saturated carboxylic acid having from 10 to 16 carbon atoms.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ-amino group of Lys^{B29} has a lipophilic substituent which is an oligo oxyethylene group comprising up to 10, preferably up to 5, oxyethylene units.

In another preferred embodiment, the invention relates to a human insulin derivative in which the ϵ-amino group of Lys^{B29} has a lipophilic substituent which is an oligo oxypropylene group comprising up to 10, preferably up to 5, oxypropylene units.

In another preferred embodiment, the invention relates to a human insulin derivative in which each insulin hexamer binds 2 Zn^{2+} ions.

In another preferred embodiment, the invention relates to a human insulin derivative in which each insulin hexamer binds 3 Zn^{2+} ions.

In another preferred embodiment, the invention relates to a human insulin derivative in which each insulin hexamer binds 4 Zn^{2+} ions.

In another preferred embodiment, the invention relates to the use of a human insulin derivative according to the invention for the preparation of a medicament for treating diabetes.

In another preferred embodiment, the invention relates to a pharmaceutical composition for the treatment of diabetes in a patient in need of such a treatment comprising a therapeutically effective amount of a human insulin derivative according to the invention together with a pharmaceutically acceptable carrier.

In another preferred embodiment, the invention relates to a pharmaceutical composition for the treatment of diabetes in a patient in need of such a treatment comprising a therapeutically effective amount of a human insulin derivative according to the invention, in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

In another preferred embodiment, the invention relates to a pharmaceutical composition comprising a human insulin derivative according to the invention which is soluble at physiological pH values.

In another preferred embodiment, the invention relates to a pharmaceutical composition comprising a human insulin derivative according to the invention which is soluble at pH values in the interval from about 6.5 to about 8.5.

In another preferred embodiment, the invention relates to a protracted pharmaceutical composition comprising a human insulin derivative according to the invention.

In another preferred embodiment, the invention relates to a pharmaceutical composition which is a solution containing from about 120 nmol/ml to about 1200 nmol/ml, preferably about 600 nmol/ml of a human insulin derivative according to the invention.

In another preferred embodiment, the invention relates to a method of treating diabetes in a patient in need of such a treatment comprising administering to the patient a therapeutically effective amount of an insulin derivative according to this invention together with a pharmaceutically acceptable carrier.

In another preferred embodiment, the invention relates to a method of treating diabetes in a patient in need of such a treatment comprising administering to the patient a therapeutically effective amount of an insulin derivative according to this invention, in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

Examples of preferred human insulin derivatives according to the present invention in which no Zn^{2+} ions are bound are the following:

$N^{\epsilon B29}$-tridecanoyl des(B30) human insulin,
$N^{\epsilon B29}$-tetradecanoyl des(B30) human insulin,
$N^{\epsilon B29}$-decanoyl des(B30) human insulin,
$N^{\epsilon B29}$-dodecanoyl des(B30) human insulin,
$N^{\epsilon B29}$-tridecanoyl Gly^{A21} des(B30) human insulin,
$N^{\epsilon B29}$-tetradecanoyl Gly^{A21} des(B30) human insulin,
$N^{\epsilon B29}$-decanoyl Gly^{A21} des(B30) human insulin,
$N^{\epsilon B29}$-dodecanoyl Gly^{A21} des(B30) human insulin,
$N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-tridecanoyl Ala^{A21} des(B30) human insulin,
$N^{\epsilon B29}$-tetradecanoyl Ala^{A21} des(B30) human insulin,
$N^{\epsilon B29}$-decanoyl Ala^{A21} des(B30) human insulin,
$N^{\epsilon B29}$-dodecanoyl Ala^{A21} des(B30) human insulin,
$N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-tridecanoyl Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-tetradecanoyl Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-decanoyl Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-dodecanoyl Gln^{B3} des(B30) human insulin,
$N^{\epsilon B29}$-tridecanoyl Gly^{A21} human insulin,

$N^{\epsilon B29}$-tetradecanoyl Gly^{A21} human insulin,
$N^{\epsilon B29}$-decanoyl Gly^{A21} human insulin,
$N^{\epsilon B29}$-dodecanoyl Gly^{A21} human insulin,
$N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} human insulin,
$N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} human insulin,
$N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} human insulin,
$N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} human insulin,
$N^{\epsilon B29}$-tridecanoyl Ala^{A21} human insulin,
$N^{\epsilon B29}$-tetradecanoyl Ala^{A21} human insulin,
$N^{\epsilon B29}$-decanoyl Ala^{A21} human insulin,
$N^{\epsilon B29}$-dodecanoyl Ala^{A21} human insulin,
$N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} human insulin,
$N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} human insulin,
$N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} human insulin,
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$N^{\epsilon B29}$-tridecanoyl Gln^{B3} human insulin,
$N^{\epsilon B29}$-tetradecanoyl Gln^{B3} human insulin,
$N^{\epsilon B29}$-decanoyl Gln^{B3} human insulin,
$N^{\epsilon B29}$-dodecanoyl Gln^{B3} human insulin,
$N^{\epsilon B29}$-tridecanoyl Glu^{B30} human insulin,
$N^{\epsilon B29}$-tetradecanoyl Glu^{B30} human insulin,
$N^{\epsilon B29}$-decanoyl Glu^{B30} human insulin,
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$N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin,
$N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin,
$N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin,
$N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin,
$N^{\epsilon B29}$-tridecanoyl Ala^{A21} Glu^{B30} human insulin,
$N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Glu^{B30} human insulin,
$N^{\epsilon B29}$-decanoyl Ala^{A21} Glu^{B30} human insulin,
$N^{\epsilon B29}$-dodecanoyl Ala^{A21} Glu^{B30} human insulin,
$N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin,
$N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin,
$N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin,
$N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin,
$N^{\epsilon B29}$-tridecanoyl Gln^{B3} Glu^{B30} human insulin,
$N^{\epsilon B29}$-tetradecanoyl Gln^{B3} Glu^{B30} human insulin,
$N^{\epsilon B29}$-decanoyl Gln^{B3} Glu^{B30} human insulin and
$N^{\epsilon B29}$-dodecanoyl Gln^{B3} Glu^{B30} human insulin.

Examples of preferred human insulin derivatives according to the present invention in which two Zn^{2+} ions are bound per insulin hexamer are the following:

($N^{\epsilon B29}$-tridecanoyl des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gln^{B3} des(B30) human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl human insulin)$_6$, $2Zn^{+}$,
($N^{\epsilon B29}$-dodecanoyl human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gln^{B3} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gln^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,

($N^{\epsilon B29}$-tridecanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$ and
($N^{\epsilon B29}$-dodecanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $2Zn^{2+}$.

Examples of preferred human insulin derivatives according to the present invention in which three Zn^{2+} ions are bound per insulin hexamer are the following:

($N^{\epsilon B29}$-tridecanoyl des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gln^{B3} des(B30) human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gln^{B3} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$ and
($N^{\epsilon B29}$-dodecanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $3Zn^{2+}$.

Examples of preferred human insulin derivatives according to the present invention in which four Zn^{2+} ions are bound per insulin hexamer are the following:

($N^{\epsilon B29}$-tridecanoyl des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gln^{B3} des(B30) human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl human insulin)$_6$, $4Zn^{2+}$,

($N^{\epsilon B29}$-decanoyl human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gln^{B3} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Gly^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Ala^{A21} $GlnB^3$ Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-dodecanoyl Ala^{A21} Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tridecanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-tetradecanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$,
($N^{\epsilon B29}$-decanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$ and
($N^{\epsilon B29}$-dodecanoyl Gln^{B3} Glu^{B30} human insulin)$_6$, $4Zn^{2+}$.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is further illustrated with reference to the appended drawings wherein

FIG. **1** shows the construction of the plasmid pEA5.3.2;

FIG. **2** shows the construction of the plasmid pEA108; and

FIG. **3** shows the construction of the plasmid pEA113.

DETAILED DESCRIPTION OF THE INVENTION

Terminology

The three letter codes and one letter codes for the amino acid residues used herein are those stated in J. Biol. Chem. 243, p. 3558 (1968).

In the DNA sequences, A is adenine, C is cytosine, G is guanine, and T is thymine.

The following acronyms are used: DMSO for dimethyl sulphoxide, DMF for dimethylformamide, Boc for tert-butoxycarbonyl, RP-HPLC for reversed phase high performance liquid chromatography, X-OSu is an N-hydroxysuccinimid ester, X is an acyl group, and TFA for trifluoroacetic acid.

Preparation of Lipophilic Insulin Derivatives

The insulin derivatives according to the present invention can be prepared i.a. as described in the following:

1. Insulin Derivatives Featuring in Position B30 an Amino Acid Residue Which can be Coded for by the Genetic Code e.g. Threonine (Human Insulin) or Alanine (Porcine Insulin)

1.1 Starting from Human Insulin

Human insulin is treated with a Boc-reagent (e.g. di-tert-butyl dicarbonate) to form (A1,B1)-diBoc human insulin, i.e., human insulin in which the N-terminal end of both chains are protected by a Boc-group. After an optional purification, e.g. by HPLC, an acyl group is introduced in the ϵ-amino group of Lys^{B29} by allowing the product to react with a N-hydroxysuccinimide ester of the formula X-OSu wherein X is the acyl group to be introduced. In the final step, TFA is used to remove the Boc-groups and the product, $NE^{68\ B29}$-X human insulin, is isolated.

1.2 Starting from a Single Chain Insulin Precursor

A single chain insulin precursor, extended in position B1 with an extension (Ext) which is connected to B1 via an arginine residue and in which the bridge from B30 to A1 is an arginine residue, i.e. a compound of the general formula Ext-Arg-B(1–30)-Arg-A(1–21), can be used as starting material. Acylation of this starting material with a N-hydroxysuccinimide ester of the general formula X-OSu wherein X is an acyl group, introduces the acyl group X in the ϵ-amino group of Lys^{B29} and in the N-terminal amino group of the precursor. On treating this acylated precursor of the formula ($N^{\epsilon B29}$-X),X-Ext-Arg-B(1–30)-Arg-A(1–21) with trypsin in a mixture of water and a suitable water-miscible organic solvent, e.g. DMF, DMSO or a lower alcohol, an intermediate of the formula ($N^{\epsilon B29}$-X),Arg^{B31} insulin is obtained. Treating this intermediate with carboxypeptidase B yields the desired product, ($N^{\epsilon B29}$-X) insulin.

2. Insulin Derivatives with no Amino Acid Residue in Position B30, i.e. des(B30) Insulins

2.1 Starting from Human Insulin or Porcine Insulin

On treatment with carboxypeptidase A in ammonium buffer, human insulin and porcine insulin both yield des (B30) insulin. After an optional purification, the des(B30) insulin is treated with a Boc-reagent (e.g. di-tert-butyl dicarbonate) to form (A1,B1)-diBoc des(B30) insulin, i.e., des(B30) insulin in which the N-terminal end of both chains are protected by a Boc-group. After an optional purification, e.g. by HPLC, an acyl group is introduced in the ϵ-amino group of Lys^{B29} by allowing the product to react with a N-hydroxysuccinimide ester of the formula X-OSu wherein X is the acyl group to be introduced. In the final step, TFA is used to remove the Boc-groups and the product, ($N^{\epsilon B29}$-X) des(B30) insulin, is isolated.

2.2 Starting from a Single Chain Human Insulin Precursor

A single chain human insulin precursor, which is extended in position B1 with an extension (Ext) which is connected to

B1 via an arginine residue and which has a bridge from B30 to A1 can be a useful starting material. Preferably, the bridge is a peptide of the formula Y_n-Arg, where Y is a codable amino acid except lysine and arginine, and n is zero or an integer between 1 and 35. When n>1, the Y's may designate different amino acids. Preferred examples of the bridge from B30 to A1 are: AlaAlaArg, SerArg, SerAspAspAlaArg and Arg (European Patent No. 163529). Treatment of such a precursor of the general formula Ext-Arg-B(1–30)-Y_n-Arg-A(1–21) with a lysyl endopeptidase, e.g. *Achromobacter lyticus* protease, yields Ext-Arg-B(1–29) Thr-Y_n-Arg-A (1–21) des(B30) insulin. Acylation of this intermediate with a N-hydroxysuccinimide ester of the general formula X-OSu wherein X is an acyl group, introduces the acyl group X in the ϵ-amino group of Lys^{B29}, and in the N-terminal amino group of the A-chain and the B-chain to give ($N^{\epsilon B29}$-X) X-Ext-Arg-B(1–29) X-Thr-Y_n-Arg-A(1–21) des(B30) insulin. This intermediate on treatment with trypsin in mixture of water and a suitable organic solvent, e.g. DMF, DMSO or a lower alcohol, gives the desired derivative, ($N^{\epsilon B29}$-X) des (B30) human insulin.

Data on $N^{\epsilon B29}$ Modified Insulins

Certain experimental data on $N^{\epsilon B29}$ modified insulins are given in Table 1.

(prolongation), see p. 211 in Markussen et al., Protein Engineering 1 (1987) 205–213. The formula has been scaled to render a value of 100 with bovine ultralente insulin and a value of 0 with Actrapid® insulin (Novo Nordisk A/S, 2880 Bagsvaerd, Denmark).

The insulin derivatives listed in Table 1 were administered in solutions containing 3 Zn^{2+} per insulin hexamer, except those specifically indicated to be Zn-free.

For the very protracted analogues the rabbit model is inadequate because the decrease in blood glucose from initial is too small to estimate the index of protraction. The prolongation of such analogues is better characterized by the disappearance rate in pigs. $T_{50\%}$ is the time when 50% of the A14 Tyr(^{125}I) analogue has disappeared from the site of injection as measured with an external γ-counter (Ribel, U et al., The Pig as a Model for Subcutaneous Absorption in Man. In: M. serrano-Rios and P. J. Lefebre (Eds): Diabetes 1985; Proceedings of the 12th Congress of the International Diabetes Federation, Madrid, Spain, 1985 (Excerpta Medica, Amsterdam, (1986) 891–96).

In Table 2 are given the $T_{50\%}$ values of a series of very protracted insulin analogues. The analogues were administered in solutions containing 3 Zn^{2+} per insulin hexamer.

TABLE 1

Insulin Derivative *)	Relative Lipophilicity	Blood glucose, % of initial 1 h	2 h	4 h	6 h	Index of protraction
$N^{\epsilon B29}$-benzoyl insulin	1.14					
$N^{\epsilon B29}$-phenylacetyl insulin (Zn-free)	1.28	55.4	58.9	88.8	90.1	10
$N^{\epsilon B29}$-cyclohexylacetyl insulin	1.90	53.1	49.6	66.9	81.1	28
$N^{\epsilon B29}$-cyclohexylpropionyl insulin	3.29	55.5	47.6	61.5	73.0	39
$N^{\epsilon B29}$-cyclohexylvaleroyl insulin	9.87	65.0	58.3	65.7	71.0	49
$N^{\epsilon B29}$-octanoyl insulin	3.97	57.1	54.8	69.0	78.9	33
$N^{\epsilon B29}$-decanoyl, des-(B30) insulin	11.0	74.3	65.0	60.9	64.1	65
$N^{\epsilon B29}$-decanoyl insulin	12.3	73.3	59.4	64.9	68.0	60
$N^{\epsilon B29}$-undecanoyl, des-(B30) insulin	19.7	88.1	80.0	72.1	72.1	80
$N^{\epsilon B29}$-lauroyl, des-(B30) insulin	37.0	91.4	90.0	84.2	83.9	78
$N^{\epsilon B29}$-myristoyl insulin	113	98.5	92.0	83.9	84.5	97
$N^{\epsilon B29}$-choloyl insulin	7.64	58.2	53.2	69.0	88.5	20
$N^{\epsilon B29}$-7-deoxycholoyl insulin (Zn-free)	24.4	76.5	65.2	77.4	87.4	35
$N^{\epsilon B29}$-lithocholoyl insulin (Zn-free)	51.6	98.3	92.3	100.5	93.4	115
$N^{\epsilon B29}$-4-benzoyl-phenylalanyl insulin	2.51	53.9	58.7	74.4	89.0	14
$N^{\epsilon B29}$-3,5-diiodotyrosyl insulin	1.07	53.9	48.3	60.8	82.1	27
$N^{\epsilon B29}$-L-thyroxyl insulin	8.00					

*) 3 Zn^{2+}/insulin hexamer except where otherwise indicated.

The lipophilicity of an insulin derivative relative to human insulin, k'_{rel}, was measured on a LiChrosorb RP18 (5 μm, 250×4 mm) HPLC column by isocratic elution at 40° C. using mixtures of A) 0.1 M sodium phosphate buffer, pH 7.3, containing 10% acetonitrile, and B) 50% acetonitrile in water as eluents. The elution was monitored by following the UV absorption of the eluate at 214 nm. Void time, t_0, was found by injecting 0.1 mM sodium nitrate. Retention time for human insulin, t_{human}, was adjusted to at least $2t_0$ by varying the ratio between the A and B solutions. $k'_{rel}=(t_{derivative}-t_0)/(t_{human}-t_0)$.

The degree of prolongation of the blood glucose lowering effect was studied in rabbits. Each insulin derivative was tested by subcutaneous injection of 12 nmol thereof in each of six rabbits in the single day retardation test. Blood sampling for glucose analysis was performed before injection and at 1, 2, 4 and 6 hours after injection. The glucose values found are expressed as percent of initial values. The Index of Protraction, which was calculated from the blood glucose values, is the scaled Index of Protraction

TABLE 2

Derivative of Human Insulin 600 μM, 3 Zn^{2+}/hexamer, phenol 0.3%, glycerol 1.6%, pH 7.5	Relative hydrophobicity k'_{rel}	Subcutaneous disappearance in pigs $T_{50\%}$, hours
$N^{\epsilon B29}$-decanoyl des(B30) insulin	11.0	5.6
$N^{\epsilon B29}$-undecanoyl des(B30) insulin	19.7	6.9
$N^{\epsilon B29}$-lauroyl des(B30) insulin	37	10.1
$N^{\epsilon B29}$-tridecanoyl des(B30) insulin	65	12.9
$N^{\epsilon B29}$-myristoyl des(B30) insulin	113	13.8
$N^{\epsilon B29}$-palmitoyl des(B30) insulin	346	12.4
$N^{\epsilon B29}$-2-succinyl-amido myristic acid insulin	10.5	13.6
$N^{\epsilon B29}$-myristoyl insulin	113	11.9
$N^{\epsilon B29}$-2-succinyl-amido palmitic acid insulin	420	20.1
$N^{\epsilon B29}$-myristoyl-α-glutamyl des(B30) insulin	23.7	8.8
$N^{\epsilon B29}$-myristoyl-α-glutamyl-glycyl des(B30) insulin	20.0	11.9

TABLE 2-continued

Derivative of Human Insulin 600 μM, 3 Zn^{2+}/hexamer, phenol 0.3%, glycerol 1.6%, pH 7.5	Relative hydrophobicity k'_{rel}	Subcutaneous disappearance in pigs $T_{50\%}$, hours
$N^{\epsilon B29}$-lithocholoyl-α-glutamyl des(B30) insulin	12.5	14.3
Human NPH		10

Solubility

The solubility of all the $N^{\epsilon B29}$ modified insulins mentioned in Table 1, which contain 3 Zn^{2+} ions per insulin hexamer, exceeds 600 nmol/ml in a neutral (pH 7.5), aqueous, pharmaceutical formulation which further comprises 0.3% phenol as preservative, and 1.6% glycerol to achieve isotonicity. 600 nmol/ml is the concentration of human insulin found in the 100 IU/ml compositions usually employed in the clinic.

The ϵ-B29 amino group can be a component of an amide bond, a sulphonamide bond, a carbamide, a thiocarbamide, or a carbamate. The lipophilic substituent carried by the ϵ-B29 amino group can also be an alkyl group.

Pharmaceutical compositions containing a human insulin derivative according to the present invention may be administered parenterally to patients in need of such a treatment. Parenteral administration may be performed by subcutaneous, intramuscular or intravenous injection by means of a syringe, optionally a pen-like syringe. Alternatively, parenteral administration can be performed by means of an infusion pump. A further option is a composition which may be a powder or a liquid for the administration of the human insulin derivative in the form of a nasal spray.

The injectable human insulin compositions of the invention can be prepared using the conventional techniques of the pharmaceutical industry which involves dissolving and mixing the ingredients as appropriate to give the desired end product.

Thus, according to one procedure, the human insulin derivative is dissolved in an amount of water which is somewhat less than the final volume of the composition to be prepared. An isotonic agent, a preservative and a buffer is added as required and the pH value of the solution is adjusted—if necessary—using an acid, e.g. hydrochloric acid, or a base, e.g. aqueous sodium hydroxide as needed. Finally, the volume of the solution is adjusted with water to give the desired concentration of the ingredients.

Examples of isotonic agents are sodium chloride, mannitol and glycerol.

Examples of preservatives are phenol, m-cresol, methyl p-hydroxybenzoate and benzyl alcohol.

Examples of suitable buffers are sodium acetate and sodium phosphate.

A composition for nasal administration of an insulin derivative according to the present invention may, for example, be prepared as described in European Patent No. 272097 (to Novo Nordisk A/S).

The insulin compositions of this invention can be used in the treatment of diabetes. The optimal dose level for any patient will depend on a variety of factors including the efficacy of the specific human insulin derivative employed, the age, body weight, physical activity, and diet of the patient, on a possible combination with other drugs, and on the severity of the case of diabetes. It is recommended that the daily dosage of the human insulin derivative of this invention be determined for each individual patient by those skilled in the art in a similar way as for known insulin compositions.

Where expedient, the human insulin derivatives of this invention may be used in mixture with other types of insulin, e.g. human insulin or porcine insulin or insulin analogues with a more rapid onset of action. Examples of such insulin analogues are described e.g. in the European patent applications having the publication Nos. EP 214826 (Novo Nordisk A/S), EP 375437 (Novo Nordisk A/S) and EP 383472 (Eli Lilly & Co.).

The present invention is further illustrated by the following examples which, however, are not to be construed as limiting the scope of protection. The features disclosed in the foregoing description and in the following examples may, both separately and in any combination thereof, be material for realizing the invention in diverse forms thereof.

EXAMPLES

Plasmids and DNA Material

All expression plasmids are of the cPOT type. Such plasmids are described in EP patent application No. 171 142 and are characterized in containing the *Schizosaccharomyces pombe* triose phosphate isomerase gene (POT) for the purpose of plasmid selection and stabilization. A plasmid containing the POT-gene is available from a deposited *E. coli* strain (ATCC 39685). The plasmids furthermore contain the *S. cerevisiae* triose phosphate isomerase promoter and terminator (P_{TPI} and T_{TPI}). They are identical to pMT742 (Egel-Mitani, M. et al., *Gene* 73 (1988) 113–120) (see FIG. 1) except for the region defined by the ECoRI-Xbal restriction sites encompassing the coding region for signal/leader/product.

Synthetic DNA fragments were synthesized on an automatic DNA synthesizer (Applied Biosystems model 380A) using phosphoramidite chemistry and commercially available reagents (Beaucage, S. L. and Caruthers, M. H., *Tetrahedron Letters* 22 (1981) 1859–1869).

All other methods and materials used are common state of the art knowledge (see, e.g. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory Press, New York, 1989).

Analytical

Molecular masses of the insulins prepared were obtained by MS (mass spectroscopy), either by PDMS (plasma desorption mass spectrometry) using a Bio-Ion 20 instrument (Bio-Ion Nordic AB, Uppsala, Sweden) or by ESMS (electrospray mass spectrometry) using an API III Biomolecular Mass Analyzer (Perkin-Elmer Sciex Instruments, Thornhill, Canada).

Example 1

Synthesis of Ala^{A21} Asp^{B3} Human Insulin Precursor from Yeast Strain yEA002 Using the LaC212spx3 Signal/Leader

The following oligonucleotides were synthesized:

#98 5'-TGGCTAAGAGATTCGTTGACCAACACTTGTGCGGTTCTCACTTGGTTGAA (SEQ ID NO:3)

GCTTTGTACTTGGTTTGTGGTGAAAGAGGTTTCTTCTACACTCCAAAGTCTGA

CGACGCT-3' (Asp^{B3})

#128 5'-CTGCGGGCTGCGTCTAAGCACAGTAGTTTTCCAATTGGTACAAAGAACAG (SEQ ID NO:4)

ATAGAAGTACAACATTGTTCAACGATACCCTTAGCGTCGTCAGACTTTGG-3' (Ala^{A21})

#126 5'-GTCGCCATGGCTAAGAGATTCGTTG-3' (Asp^{B3}) (SEQ ID NO:5)

#16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3' (SEQ ID NO:6)

The following Polymerase Chain Reaction (PCR) was performed using the Gene Amp PCR reagent kit (Perkin Elmer, 761 Main Avewalk, Conn. 06859, U.S.A.) according to the manufacturer's instructions. In all cases, the PCR mixture was overlayed with 100 μl of mineral oil (Sigma Chemical Co., St. Louis, Mo., U.S.A.).
2.5 μl of oligonucleotide #98 (2.5 pmol)
2.5 μl of oligonucleotide #128 (2.5 pmol)
10 μl of 10× PCR buffer
16 μl of dNTP mix
0.5 μl of Taq enzyme
58.5 μl of water

One cycle was performed: 94° C. for 45 sec., 49° C. for 1 min, 72° C. for 2 min.

Subsequently, 5 μl of oligonucleotides #16 and #126 was added and 15 cycles were performed: 94° C. for 45 sec., 45° C. for 1 min, 72° C. for 1.5 min. The PCR mixture was loaded onto a 2.5% agarose gel and subjected to electrophoresis using standard techniques (Sambrook et al., Molecular cloning, Cold Spring Harbour Laboratory Press, 1989). The resulting DNA fragment was cut out of the agarose gel and isolated using the Gene Clean Kit (Bio 101 Inc., PO BOX 2284, La Jolla, Calif., 92038, U.S.A.) according to the manufacturer's instructions. The purified PCR DNA fragment was dissolved in 10 μl of water and restriction endonuclease buffer and cut with the restriction endonucleases NcoI and XbaI according to standard techniques, run on a 2.5% agarose gel and purified using the Gene Clean Kit as described.

The plasmid pAK188 consists of a DNA sequence of 412 bp composed of a EcoRI/NcoI fragment encoding the synthetic yeast signal/leader gene LaC212spx3 (described in Example 3 of WO 89/02463) followed by a synthetic NcoI/XbaI fragment encoding the insulin precursor M15, which has a SerAspAspAlaLys bridge connecting the B29 and the A1 amino acid residues (see SEQ ID NOS. 14, 15 and 16), inserted into the EcoRI/XbaI fragment of the vector (phagemid) pBLUESCRIPT IIsk(±) (Stratagene, U.S.A.). The plasmid pAK188 is shown in FIG. 1.

The plasmid pAK188 was also cut with the restriction endonucleases NcoI and XbaI and the vector fragment of 3139 bp isolated. The two DNA fragments were ligated together using T4 DNA ligase and standard conditions (Sambrook et al., Molecular Cloning, Cold Spring Harbour Laboratory Press, 1989). The ligation mixture was transformed into a competent *E. coli* strain (R−, M+) followed by selection for ampicillin resistance. Plasmids were isolated from the resulting *E. coli* colonies using standard DNA miniprep technique (Sambrook et al., Molecular Cloning, Cold Spring Harbour Laboratory Press, 1989), checked with appropriate restrictions endonucleases i.e. EcoRI, XbaI, NcoI and HpaI. The selected plasmid was shown by DNA sequencing analyses (Sequenase, U.S. Biochemical Corp.) to contain the correct sequence for the Ala^{A21}, Asp^{B3} human insulin precursor and named pEA5.3.

The plasmid pKFN1627 is an *E. coli—S. cerevisiae* shuttle vector, identical to plasmid pKFN1003 described in EP patent No. 375718, except for a short DNA sequence upstream from the unique XbaI site. In pKFN1003, this sequence is a 178 bp fragment encoding a synthetic aprotinin gene fused in-frame to the yeast mating factor alpha 1 signal-leader sequence. In pKFN1627, the corresponding 184 bp sequence encodes the insulin precursor M15 (Glu^{B1}, Glu^{B28}) (i.e. B(1–29, Glu^{B1},Glu^{B28})-SerAspAspAlaLys-A (1–21) fused in-frame to the mating factor alpha 1 sequence (see SEQ ID NOS. 17, 18 and 19). The vector pKFN1627 is shown in FIG. 1.

pEA5.3 was cut with the restriction endonucleases EcoRI and XbaI and the resulting DNA fragment of 412 bp was isolated. The yeast expression vector pKFN1627 was cut with the restriction endonucleases NcoI and XbaI and with NcoI and EcoRI and the DNA fragment of 9273 bp was isolated from the first digestion and the DNA fragment of 1644 bp was isolated from the second. The 412 bp EcoRI/XbaI fragment was then ligated to the two other fragments, that is the 9273 bp NcoII/XbaI fragment and the 1644 bp NcoI/EcoRI fragment using standard techniques.

The ligation mixture was transformed into *E. coli* as described above. Plasmid from the resulting *E. coli* was isolated using standard techniques, and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI, Hpa I. The selected plasmid was shown by DNA sequence analysis (using the Sequenase kit as described by the manufacturer, U.S. Biochemical) to contain the correct sequence for the Ala^{A21} Asp^{B3} human insulin precursor DNA and to be inserted after the DNA encoding the LaC212spx3 signal/leader DNA. The plasmid was named pEA5.3.2 and is shown in FIG. 1. The DNA sequence encoding the LaC212spx3 signal/leader/Ala^{A21} Asp^{B3} human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 20, 21 and 22. The plasmid pEA5.3.2 was transformed into *S. cerevisiae* strain MT663 as described in European patent application having the publication No. 214826 and the resulting strain was named yEA002.

Example 2

Synthesis of Ala^{A21} Thr^{B3} Human Insulin Precursor from Yeast Strain yEA005 Using the LaC212spx3 Signal/Leader

The following oligonucleotides were synthesized:

#101 5'-TGGCTAAGAGATTCGTTACTCAACACTTGTGCGGTTCTCACTT (SEQ ID NO:7)

GGTTGAAGCTTTGTACTTGGTTTGTGGTGAAAGAGGTTTCTTCTACA

CTCCAAAGTCTGACGACGCT-3' THR^{B3})

#128 5'-CTGCGGGCTGCGTCTAAGCACAGTAGTTTTCCAATTGGTACAAA (SEQ ID NO:4)

GAACAGATAGAAGTACAACATTGTTCAACGATACCCTTAGCGTCG

TCAGACTTTGG-3'(Ala^{A21})

#15 5'-GTCGCCATGGCTAAGAGATTCGTTA-3' (Thr^{B3}) (SEQ ID NO:8)

#16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3' (SEQ ID NO:6)

The DNA encoding Ala^{A21} Thr^{B3} human insulin precursor was constructed in the same manner as described for the DNA encoding Ala^{A21} Asp^{B3} human insulin precursor in Example 1. The DNA sequence encoding the LaC212spx3 signal/leader/Ala^{A21} Thr^{B3} human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 23, 24 and 25. The plasmid pEA8.1.1 was shown to contain the desired sequence, transformed into *S. cerevisiae* strain MT663 as described in Example 1 and the resulting strain was named yEA005.

Example 3

Synthesis of Gly^{A21} Asp^{B3} Human Insulin Precursor from Yeast Strain yEA007 Using the LaC212spx3 Signal/Leader

The following oligonucleotides were synthesized:

#98 5'-TGGCTAAGAGATTCGTTGACCAACACTTGTGCGGTTCTCACTTG (SEQ ID NO:3)

GTTGAAGCTTTGTACTTGGTTTGTGGTGAAAGAGGTTTCTTCT

ACACTCCAAAGTCTGACGACGCT-3' (Asp^{B3})

#127 5'-CTGCGGGCTGCGTCTAACCACAGTAGTTTTCCAATTGGTACAA (SEQ ID NO:9)

AGAACAGATAGAAGTACAACATTGTTCAACGATACCCT

-continued

TAGCGTCGTCAGACTTTGG-3' (Gly^{A21})

#126 5'-GTCGCCATGGCTAAGAGATTCGTTG-3' (Asp^{B3}) (SEQ ID NO:5)

#16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3' (SEQ ID NO:6)

The DNA encoding Gly^{A21} Asp^{B3} human insulin precursor was constructed in the same manner as described for the DNA encoding Ala^{A21} Asp^{B3} human insulin precursor in Example 1. The DNA sequence encoding the LaC212spx3 signal/leader/Gly^{A21} Asp^{B3} human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 26, 27 and 28. The plasmid pEA1.5.6 was shown to contain the desired sequence, transformed into *S. cerevisiae* strain MT663 as described in Example 1 and the resulting strain was named yEA007.

Example 4

Synthesis of Gly^{A21} Thr^{B3} Human Insulin Precursor from Yeast Strain yEA006 Using the LaC212spx3 Signal/Leader

The following oligonucleotides were synthesized:

#101 5'-TGGCTAAGAGATTCGTTACTCAACACTTGTGCGGTTCTCACTTGGTTGAAG (SEQ ID NO:7)

CTTTGTACTTGGTTTGTGGTGAAAGAGGTTTCTTCTACACTCCAAAGTCTGACG

ACGCT-3' (Thr^{B3})

#127 5'-CTGCGGGCTGCGTCTAACCACAGTAGTTTTCCAATTGGTACAAAGAACAG (SEQ ID NO:9)

ATAGAAGTACAACATTGTTCAACGATACCCTTAGCGTCGTCAGACTTTGG-3' (Gly^{A21})

#15 5'-GTCGCCATGGCTAAGAGATTCGTTA-3' (Thr^{B3}) (SEQ ID NO:8)

#16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3' (SEQ ID NO:6)

The DNA encoding Gly^{A21} Thr^{B3} human insulin precursor was constructed in the same manner as described for the DNA encoding Ala^{A21} Asp^{B3} human insulin precursor in Example 1. The DNA sequence encoding the LaC212spx3 signal/leader/Gly^{A21} Thr^{B3} human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 29, 30 and 31. The plasmid pEA4.4.11 was shown to contain the desired DNA sequence, transformed into *S. cerevisiae* strain MT663 as described in Example 1 and the resulting strain was named yEA006.

Example 5

Synthesis of Arg^{B-1} Arg^{B31} Single Chain Human Insulin Precursor Having an N-Terminal Extension (GluGluAlaGluAlaGluAlaArg) from Yeast Strain yEA113 Using the Alpha Factor Leader

A) The following oligonucleotides were synthesized:

#220 5'-ACGTACGTTCTAGAGCCTGCGGGCTGC-3' (SEQ ID NO:10)

#263 5'-CACTTGGTTGAAGCTTTGTACTTGGTTTGTGGTGAAAGAGGTTTC (SEQ ID NO:11)

TTCTACACTCCAAAGACTAGAGGTATCGTTGAA-3'

#307 5'-GCTAACGTCGCCATGGCTAAGAGAGAAGAAGCTGAAGCTGAAGCT (SEQ ID NO:12)

AGATTCGTTAACCAACAC-3'

The following Polymerase Chain Reaction (PCR) was performed using the Gene Amp PCR reagent kit (Perkin Elmer, 761 Main Avewalk, Conn. 06859, U.S.A.) according to the manufacturer's instructions. In all cases, the PCR mixture was overlayed with 100 μl of mineral oil (Sigma Chemical Co, St. Louis, Mo., U.S.A.). The plasmid pAK220 (which is identical to pAK188) consists of a DNA sequence of 412 bp encoding the synthetic yeast signal/leader LaC212spx3 (described in Example 3 of WO 89/02463) followed by the insulin precursor M15 (see SEQ ID NOS. 14, 15 and 16) inserted into the vector (phagemid) pBLUESCRIPT IIsk(±) (Stratagene, U.S.A.).

5 μl of oligonucleotide #220 (100 pmol)
5 μl of oligonucleotide #263 (100 pmol)
10 μl of 10× PCR buffer
16 μl of dNTP mix
0.5 μl of Taq enzyme
0.5 μl of pAK220 plasmid (identical to pAK188) as template (0.2 μg of DNA)
63 μl of water

A total of 16 cycles were performed, each cycle comprising 1 minute at 95° C.; 1 minute at 40° C.; and 2 minutes at 72° C. The PCR mixture was then loaded onto a 2% agarose gel and subjected to electrophoresis using standard techniques. The resulting DNA fragment was cut out of the agarose gel and isolated using the Gene Clean kit (Bio 101 Inc., PO BOX 2284, La Jolla, Calif., 92038, U.S.A.) according to the manufacture's instructions. The purified PCR DNA fragment was dissolved in 10 μl of water and restriction endonuclease buffer and cut with the restriction endonucleases HindIII and XbaI according to standard techniques. The HindIII/XbaI DNA fragment was purified using The Gene Clean Kit as described.

The plasmid pAK406 consists of a DNA sequence of 520 bp comprising an EcoRI/HindIII fragment derived from pMT636 (described in WO 90/10075) encoding the yeast alpha factor leader and part of the insulin precursor ligated to the HindIII/XbaI fragment from pAK188 encoding the rest of the insulin precursor MI5 (see SEQ ID NOS. 32, 33 and 34) inserted into the vector cPOT. The vector pAK406 is shown in FIG. 2.

The plasmid pAK233 consists of a DNA sequence of 412 bp encoding the synthetic yeast signal/leader LaC212spx3 (described in Example 3 of WO 89/02463) followed by the gene for the insulin precursor B(1–29)-GluLysArg-A(1–21) (A21-Gly) (see SEQ ID NOS. 35, 36 and 37) inserted into the vector cPOT. The plasmid pAK233 is shown in FIG. 2.

The plasmid pAK233 was cut with the restriction endonucleases NcoI and XbaI and the vector fragment of 9273 bp isolated. The plasmid pAK406 was cut with the restriction endonucleases NcoI and HindIII and the vector fragment of 2012 bp isolated. These two DNA fragments were ligated together with the HindIII/XbaI PCR fragment using T4 DNA ligase and standard conditions. The ligation mixture was then transformed into a competent *E. coli* strain (R–, M+) followed by selection for ampicillin resistance. Plasmids were isolated from the resulting *E. coli* colonies using a standard DNA miniprep technique and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI, HindIII. The selected plasmid was shown by DNA sequencing analyses to contain the correct sequence for the Arg^{B31} single chain human insulin precursor DNA and to be inserted after the DNA encoding the *S. cerevisiae* alpha factor DNA. The plasmid was named pEA108 and is shown in FIG. 2. The DNA sequence encoding the alpha factor leader/Arg^{B31} single chain human insulin precursor complex and the amino acid sequence thereof are SEQ ID NOS. 38, 39 and 40. The plasmid pEA108 was transformed into *S. cerevisiae* strain MT663 as described in Example 1 and the resulting strain was named yEA108.

B) The following Polymerase Chain Reaction (PCR) was performed using the Gene Amp PCR reagent kit (Perkin Elmer, 761 Main Avewalk, Conn. 06859, U.S.A.) according to the manufacturer's instructions. In all cases, the PCR mixture was overlayed with 100 μl of mineral oil (Sigma Chemical Co., St. Louis, Mo., U.S.A.).

5 μl of oligonucleotide #220 (100 pmol)
5 μl of oligonucleotide #307 (100 pmol)
10 μl of 10× PCR buffer
16 μl of dNTP mix
0.5 μl of Taq enzyme
0.2 μl of pEA108 plasmid as template (0.1 ug DNA)
63 μl of water

A total of 16 cycles were performed, each cycle comprising 1 minute at 95° C.; 1 minute at 40° C.; and 2 minutes at 72° C. The PCR mixture was then loaded onto an 2% agarose gel and subjected to electrophoresis using standard techniques. The resulting DNA fragment was cut out of the agarose gel and isolated using the Gene Clean kit (Bio 101 Inc., PO BOX 2284, La Jolla, Calif., 92038, U.S.A.) according to the manufacture's instructions. The purified PCR DNA fragment was dissolved in 10 μl of water and restric-

tion endonuclease buffer and cut with the restriction endonucleases NcoI and XbaI according to standard techniques. The NcoI/XbaI DNA fragment was purified using The Gene Clean Kit as described.

The plasmid pAK401 consists of a DNA sequence of 523 bp composed of an EcoRI/NcoI fragment derived from pMT636 (described in WO 90/10075) (constructed by by introducing a NcoI site in the 3'-end of the alpha leader by site directed mutagenesis) encoding the alpha factor leader followed by a NcoI/XbaI fragment from pAK188 encoding the insulin precursor M15 (see SEQ ID NOS. 41, 42 and 43) inserted into the vector (phagemid) pBLUESCRIPT IIsk(±) (Stratagene, U.S.A.). The plasmid pAK401 is shown in FIG. **3**.

The plasmid pAK401 was cut with the restriction endonucleases NcoI and XbaI and the vector fragment of 3254 bp isolated and ligated together with the NcoI/XbaI PCR fragment. The ligation mixture was then transformed into a competent *E. coli* strain and plasmids were isolated from the resulting *E. coli* colonies using a standard DNA miniprep technique and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI. The selected plasmid, named p113A (shown in FIG. **3**), was cut with EcoRI and XbaI and the fragment of 535 bp isolated.

The plasmid pAK233 was cut with the restriction endonucleases NcoI and XbaI, and with EcoRI/NcoI and the fragments of 9273 and 1644 bp isolated. These two DNA fragments were ligated together with the EcoRI/XbaI fragment from p113A using T4 DNA ligase and standard conditions. The ligation mixture was then transformed into a competent *E. coli* strain (R−, M+) followed by selection for ampicillin resistance. Plasmids were isolated from the resulting *E. coli* colonies using a standard DNA miniprep technique and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI, HindIII. The selected plasmid was shown by DNA sequencing analyses to contain the correct sequence for the Arg^{B31} single chain human insulin precursor DNA with the N-terminal extension GluGluAlaGluAlaGluAlaArg and to be inserted after the DNA encoding the *S. cerevisiae* alpha factor DNA. The plasmid was named pEA113 and is shown in FIG. **3**. The DNA sequence encoding the alpha factor leader/Arg^{B-1} ArgB31 single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaArg) and the amino acid sequence thereof are SEQ ID NOS. 44, 45 and 46. The plasmid pEA113 was transformed into *S. cerevisiae* strain MT663 as described in Example 1 and the resulting strain was named yEA113.

Example 6

Synthesis of Arg^{B-1} Arg^{B31} Single Chain Human Insulin Precursor Having an N-Terminal Extension (GluGluAlaGluAlaGluAlaGluArg) from Yeast Strain yEA136 Using the Alpha Factor Leader

The following oligonucleotide was synthesized:

#389 5'-GCTAACGTCGCCATGGCTAAGAGAGAAGAAGCTGAAGCGAAGCTGAAAGATTCGTTAACCAACAC-3' (SEQ ID NO:13)

The following PCR was performed using the Gene Amp PCR reagent kit

5 μl of oligonucleotide #220 (100 pmol)
5 μl of oligonucleotide #389 (100 pmol)
10 μl of 10x PCR buffer
16 μl of dNTP mix
0.5 μl of Taq enzyme
0.2 μl of pEA113 plasmid as template (0.5 ug DNA)
63 μl of water

A total of 12 cycles were performed, each cycle comprising 1 minute at 95° C.; 1 minute at 37° C.; and 2 minutes at 72° C.

The DNA encoding alpha factor leader/Arg^{B-1} Arg^{B31} single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaGluArg) was constructed in the same manner as described for the DNA encoding alpha factor leader/Arg^{B-1} Arg^{B31} single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaArg) in Example 5. The plasmid was named pEA136. The DNA sequence encoding the alpha factor leader/Arg^{B-1} Arg^{B31} single chain human insulin precursor having an N-terminal extension (GluGluAlaGluAlaGluAlaGluArg) and the amino acid sequence thereof are SEQ ID NOS. 47, 48 and 49. The plasmid pEA136 was transformed into *S. cerevisiae* strain MT663 as described in Example 1 and the resulting strain was named yEA136.

Example 7

Synthesis of (A1,B1)-diBoc Human Insulin

5 g of zinc-free human insulin was dissolved in 41.3 ml of DMSO. To the solution was added 3.090 ml of acetic acid. The reaction was conducted at room temperature and initiated by addition of 565 mg of di-tert-butyl pyrocarbonate dissolved in 5.650 ml of DMSO. The reaction was allowed to proceed for 5½ hour and then stopped by addition of 250 μl of ethanolamine. The product was precipitated by addition of 1500 ml of acetone. The precipitate was isolated by centrifugation and dried in vacuum. A yield of 6.85 g material was obtained.

(A1,B1)-diBoc insulin was purified by reversed phase HPLC as follows: The crude product was dissolved in 100 ml of 25% ethanol in water, adjusted to pH 3.0 with HCl and applied to a column (5 cm diameter, 30 cm high) packed with octadecyldimethylsilyl-substituted silica particles (mean particle size 15 μm, pore size 100 Å) and equilibrated with elution buffer. The elution was performed using mixtures of ethanol and 1 mM aqueous HCl, 0.3 M KCl at a flow of 2 l/h. The insulin was eluted by increasing the ethanol content from 30% to 45%. The appropriate fraction was diluted to 20% ethanol and precipitated at pH 4.8. The precipitated material was isolated by centrifugation and dried in vacuum. Thus 1.701 g of (A1,B1)-diBoc human insulin was obtained at a purity of 94.5%.

Example 8

Synthesis of ($N^{\epsilon B29}$-benzoyl Human Insulin)$_6$, $3Zn^{2+}$

400 mg of (A1,B1)-diBoc human insulin was dissolved in 2 ml of DMSO. To the solution was added 748 μl of a mixture of N-methylmorpholine and DMSO (1:9, v/v). The reaction was conducted at 15° C. and initiated by addition of 14.6 mg of benzoic acid N-hydroxysuccinimide ester dissolved in 132 μl DMF. The reaction was stopped after 2 hours by addition of 100 ml of acetone. The precipitated material was isolated by centrifugation and dried in vacuum. 343 mg of material was collected.

The Boc protecting groups were eliminated by addition of 4 ml of TFA. The dissolved material was incubated for 30 minutes and then precipitated by addition of 50 ml of acetone. The precipitate was isolated by centrifugation and dried in vacuum.

$N^{\epsilon B29}$-benzoyl human insulin was purified by reversed phase HPLC as described in Example 7. A yield of 230 mg was obtained. Recrystallization from 15% aqueous ethanol containing 6 mM Zn^{2+} and 50 mM citrate at pH 5.5 gave crystals of the title compound which were isolated by centrifugation and dried in vacuum. The yield was 190 mg.

Molecular mass, found by MS: 5911, theory: 5911.

Example 9

Synthesis of ($N^{\epsilon B29}$-lithocholoyl Human Insulin)$_6$, $3Zn^{2+}$

400 mg of (A1,B1)-diBoc human insulin was dissolved in 2 ml of DMSO. To the solution was added 748 μl of a mixture of N-methylmorpholine and DMSO (1:9, v/v). The reaction was conducted at 15° C. and initiated by addition of 31.94 mg of lithocholic acid N-hydroxysuccinimide ester dissolved in 300 μl of DMF. The reaction was stopped after 2 hours by addition of 100 ml of acetone. The precipitated material was isolated by centrifugation and dried in vacuum. 331 mg of material was obtained.

The Boc protecting groups were eliminated by addition of 4 ml of TFA. The dissolved material was incubated for 30 minutes and then precipitated by addition of 50 ml of acetone. The precipitate was isolated by centrifugation and dried in vacuum. The yield was 376 mg.

B29-lithocholoyl insulin was purified by reversed phase HPLC as described in Example 7. A final yield of 67 mg was obtained at a purity of 94%. Recrystallization from 15% aqueous ethanol containing 6 mM Zn^{2+} and 50 mM citrate at pH 5.5 gave crystals of the title compound which were isolated by centrifugation and dried in vacuum. The yield was 49 mg.

Molecular mass, found by MS: 6160, theory: 6166.

Example 10

Synthesis of ($N^{\epsilon B29}$-decanoyl Human Insulin)$_6$, $3Zn^{2+}$

400 mg of (A1,B1)-diBoc human insulin was dissolved in 2 ml of DMSO. To the solution was added 748 μl of a mixture of N-methylmorpholine and DMSO (1:9, v/v). The reaction was conducted at 15° C. and initiated by addition of 18.0 mg of decanoic acid N-hydroxysuccinimide ester dissolved in 132 μl of DMF. The reaction was stopped after 60 minutes and the product precipitated by addition of 100 ml of acetone. The precipitated material was isolated by centrifugation and dried in vacuum. 420 mg of intermediate product was collected.

The Boc protecting groups were eliminated by addition of 4 ml of TFA. The dissolved material was incubated for 30 minutes and the product was then precipitated by addition of 50 ml of acetone. The precipitate was isolated by centrifugation and dried in vacuum. The yield of crude product was 420 mg.

The crude product was purified by reversed phase HPLC as described in Example 7. A final yield of 254 mg of the title product was obtained. The purity was 96.1%. Recrystallization from 15% aqueous ethanol containing 6 mM Zn^{2+} and 50 mM citrate at pH 5.5 gave crystals of the title compound which were isolated by centrifugation and dried in vacuum. The yield was 217 mg.

Molecular mass, found by MS: 5962, theory: 5962.

Example 11

Synthesis of des(B30) Human Insulin

Synthesis of des(B30) human insulin was carried out as described by Markussen (Methods in diabetes research, Vol. I, Laboratory methods, part B, 404–410. Ed: J. Larner and S. Phol, John Wiley & Sons, 1984). 5 g of human insulin was dissolved in 500 ml of water while the pH value of the solution was kept at 2.6 by addition of 0.5 M sulphuric acid. Subsequently, the insulin was salted out by addition of 100 g of ammonium sulphate and the precipitate was isolated by centrifugation. The pellet was dissolved in 800 ml of 0.1 M ammonium hydrogen carbonate and the pH value of the solution was adjusted to 8.4 with 1 M ammonia.

50 mg of bovine carboxypeptidase A was suspended in 25 ml of water and isolated by centrifugation. The crystals were suspended in 25 ml of water and 1 M ammonia was added until a clear solution was obtained at a final pH of 10. The carboxypeptidase solution was added to the insulin solution and the reaction was allowed to proceed for 24 hours. A few drops of toluene were added to act as preservative during the reaction.

After 24 hours the des(B30) human insulin was crystallized by successive addition of 80 g of sodium chloride while the solution was stirred. The pH value was then adjusted to 8.3 and the crystallization was allowed to proceed for 20 hours with gentle stirring. The crystals were isolated on a 1.2 μl filter, washed with 250 ml of ice cold 2-propanol and finally dried in vacuum.

Example 12

Synthesis of (A1,B1)-diBoc des(B30) Human Insulin

The title compound was synthesized by a method similar to that described in Example 7, using des(B30) porcine insulin as the starting material. The crude product was precipitated by acetone and dried in vacuum. The (A1,B1)-diBoc des(B30) human insulin was purified by reversed phase HPLC as described in Example 7.

Example 13

Synthesis of $N^{\epsilon B29}$-decanoyl des(B30) Human Insulin

400 mg of (A1,B1)-diBoc des(B30) human insulin was used as starting material for the synthesis of $N^{\epsilon B29}$-decanoyl des(B30) human insulin, following the procedure described in Example 10. The crude product was precipitated by acetone, dried in vacuum and deprotected using TFA. The resulting product was precipitated by acetone and dried in vacuum. $N^{\epsilon B29}$-decanoyl des(B30) human insulin was then purified by reversed phase HPLC as described in Example 10.

Molecular mass, found by MS: 5856, theory: 5861.

Example 14

Synthesis of $N^{\epsilon B29}$-dodecanoyl des(B30) Human Insulin

a. Immobilization of *A. lyticus* Protease

13 mg of *A. lyticus* protease, dissolved in 5 ml of aqueous 0.2 M $NaHCO_3$ buffer, pH 9.4, was mixed with 4 ml of settled MiniLeak® Medium gel, which had been washed with the same buffer (MiniLeak is a divinylsulfone activated Sepharose CL 6B, obtained from KemEnTec, Copenhagen). The gel was kept in suspension by gentle stirring for 24 hours at room temperature. Then, the gel was isolated by filtration, washed with water, and suspended in 20 ml of 1 M ethanolamine buffer, pH 9.4, and kept in suspension for 24 hours at room temperature. Finally, the gel was washed

with water followed by 0.1 M acetic acid and stored at 4° C. The enzyme activity in the filtrate was 13% of that in the initial solution, indicating a yield in the immobilization reaction of about 87%.

b. Immobilization of Porcine Trypsin

Porcine trypsin was immobilized to MiniLeak® Low to a degree of substitution of 1 mg per ml of gel, using the conditions described above for immobilization of *A. lyticus*.

c. Synthesis of Glu(GluAla)$_3$Arg-B(1–29), ThrArg-A(1–21) Insulin Using Immobilized *A. lyticus* Protease

To 200 mg of Glu(GluAla)$_3$Arg-B(1–29)-ThrArg-A (1–21) single-chain human insulin precursor, dissolved in 20 ml of 0.1 M $NaHCO_3$ buffer, pH 9.0, was added 4 ml of the gel carrying the immobilized *A. lyticus* protease. After the gel had been kept in suspension in the reaction mixture for 6 hours at room temperature the hydrolysis was complete, rendering Glu(GluAla)$_3$-Arg-B(1–29), ThrArg-A(1–21) human insulin (the reaction was followed by reversed phase HPLC). After the hydrolysis, the gel was removed by filtration. To the filtrate was added 5 ml of ethanol and 15 μl of 1 M $ZnCl_2$ and the pH was adjusted to 5.0 using HCl. The precipitation of the product was completed on standing overnight at 4° C. with gentle stirring. The product was isolated by centrifugation. After one washing with 1 ml of ice cold 20% ethanol and drying in vacuo the yield was 190 mg.

d. Synthesis of $N^{\alpha A1}$,$N^{\alpha B1}$,$N^{\epsilon B29}$-tridodecanoyl Glu (GluAla)$_3$Arg-B(1–29), Thr-Arg-A(1–21) Human Insulin Using Dodecanoic Acid N-hydroxysuccinimide Ester

190 mg (30 μmol) of Glu(GluAla)$_3$Arg-B(1–29),ThrArg-A(1–21) insulin was dissolved in 1 ml of DMSO and 1.05 ml of a 0.572 M solution of N,N-diisopropylethylamine in DMF. The solution was cooled to 15° C. and 36 mg (120 μmol) of dodecanoic acid N-hydroxysuccinimide ester dissolved in 0.6 ml of DMSO was added. The reaction was completed within 24 hours. The lipophilic title compound was not isolated.

e. Synthesis of $N^{\epsilon B29}$-dodecanoyl des(B30) Insulin

The product from the previous step, d., contained in approximately 2.65 ml of DMSO/DMF/N,N-diisopropylethylamine was diluted with 10.6 ml of a 50 mM glycine buffer comprising 20% ethanol and the pH adjusted to 10 with NaOH. After standing for 1 hour at room temperature 1 ml of MiniLeak gel, carrying 1 mg of immobilized trypsin per ml of gel, was added. The reaction mixture was stirred gently for 48 hours at room temperature. In order to isolate the desired product, the reaction mixture was applied to a reversed phase HPLC column (5 cm in diameter, 30 cm high), packed with octadecyldimethylsilyl-substituted silica particles (mean particle size 15 μm, pore size 100 Å). For the elution was used 20 mM Tris/HCl buffers, adjusted to pH 7.7 and comprising an increasing concentration of ethanol, from 40% to 44% (v/v), at a rate of 2000 ml/h. The major peak eluting at about 43–44% of ethanol contained the title compound. The fractions containing the major peak were pooled, water was added to reduce the ethanol concentration to 20% (v/v), and the pH was adjusted to 5.5. The solution was left overnight at −20° C., whereby the product precipitated. The precipitate was isolated by centrifugation at −8° C. and dried in vacuo. The yield of the title compound was 90 mg.

Molecular mass, found by MS: 5892, theory: 5890.

Example 15

Synthesis of $N^{\epsilon B29}$-(N-myristoyl-α-glutamyl) Human Insulin

500 mg of (A1,B1)-diBoc human insulin was dissolved in 2.5 ml of DMSO and 428 μl of ethyl diisopropylamine, diluted with 2.5 ml of DMSO/DMF 1/1 (v/v), was added. The temperature was adjusted to 15° C. and 85 mg of N-myristoyl-Glu(OBut) N-hydroxysuccinimide ester, dissolved in 2.5 ml of DMSO/DMF 1/1 (v/v), was added. After 30 min the reaction mixture was poured into 60 ml of water, the pH adjusted to 5 and the precipitate isolated by centrifugation. The precipitate was dried in vacuo. The dried reaction mixture was dissolved in 25 ml of TFA, and the solution was left for 30 min at room temperature. The TFA was removed by evaporation in vacuo. The gelatinous residue was dissolved in 60 ml of water and the pH was adjusted to 11.2 using concentrated ammonia. The title compound was crystallized from this solution by adjustment of the pH to 8.51 using 6 N HCl. The product was isolated by centrifugation, washed once by 10 ml of water, and dried in vacuo. Yield 356 mg. Purity by HPLC 94%.

The product of this example is thus human insulin wherein the ε-amino group of Lys^{B29} has a substituent of the following structure: $CH_3(CH_2)_{12}CONHCH(CH_2CH_2COOH)CO$—.

Molecular mass, found by MS: 6146, theory: 6148.

Example 16

Synthesis of $N^{\epsilon B29}$-undecanoyl-des(B30) Human Insulin

The title compound was synthesized analogously to $N^{\epsilon B29}$-dodecanoyl des(B30) human insulin as described in Example 14, by using undecanoic acid N-hydroxysuccinimide ester instead of dodecanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 5876, theory: 5876.

Example 17

Synthesis of $N^{\epsilon B29}$-tridecanoyl des(B30) Human Insulin

The title compound was synthesized analogously to $N^{\epsilon B29}$-dodecanoyl des(B30) human insulin as described in Example 14, by using tridecanoic acid N-hydroxysuccinimide ester instead of dodecanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 5899, theory: 5904.

Example 18

Synthesis of $N^{\epsilon B29}$-myristoyl des(B30) Human Insulin

The title compound was synthesized analogously to $N^{\epsilon B29}$-dodecanoyl des(B30) human insulin as described in Example 14, by using myristic acid N-hydroxysuccinimide ester instead of dodecanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 5923, theory: 5918.

Example 19

Synthesis of $N^{\epsilon B29}$-palmitoyl des(B30) Human Insulin

The title compound was synthesized analogously to $N^{\epsilon B29}$-dodecanoyl des(B30) human insulin as described in Example 14, by using palmitic acid N-hydroxysuccinimide ester instead of dodecanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 5944, theory: 5946.

Example 20

Synthesis of $N^{\epsilon B29}$-suberoyl-D-thyroxine Human Insulin

a. Preparation of N-(succinimidylsuberoyl)-D-thyroxine

Disuccinimidyl suberate (1.0 g, Pierce) was dissolved in DMF (50 ml), and D-thyroxine (2.0 g, Aldrich) was added with stirring at 20° C. The thyroxine slowly dissolved, and after 20 hours the solvent was removed by evaporation in vacuo. The oily residue was crystallized from 2-propanol to yield 0.6 g of N-(succinimidylsuberoyl)-D-thyroxine, m.p. 128–133° C.

b. Reaction of (A1,B1)-diBoc Human Insulin with N-(succinimidylsuberoyl)-D-thyroxine

(A1,B1)-diBoc human insulin (200 mg) was dissolved in dry DMF (10 ml) by addition of triethylamine (20 μl) at room temperature. Then, N-(succinimidylsuberoyl)-D-thyroxine (80 mg) was added. The reaction was monitored by reversed phase HPLC and when the reaction was about 90% complete, the solvent was removed in vacuo. To the evaporation residue, anhydrous trifluoroacetic acid (5 ml) was added, and the solution was kept for 1 hour at room temperature. After removal of the trifluoroacetic acid in vacuo, the residue was dissolved in a mixture of 1M acetic acid (5 ml) and acetonitrile (1.5 ml), purified by preparative reversed phase HPLC and desalted on a PD-10 column. The yield of $N^{\epsilon B29}$-suberoyl-D-thyroxine human insulin was 50 mg.

The product of this example is thus human insulin wherein the ε-amino group of Lys^{B29} has a substituent of the following structure: Thyrox-$CO(CH_2)_6CO$—, wherein Thyrox is thyroxine which is bound to the octanedioic acid moiety via an amide bond to its α-amino group.

Molecular mass of the product found by MS: 6724, theory: 6723.

Example 21

Synthesis of $N^{\epsilon B29}$-(2-succinylamido)myristic acid Human Insulin

a. Preparation of α-aminomyristic acid methyl ester,HCl

To methanol (5 ml, Merck) at −10° C., thionyl chloride (0.2 ml, Aldrich) was added dropwise while stirring vigorously. Then, α-aminomyristic acid (0.7 g, prepared from the α-bromo acid by reaction with ammonia) was added. The reaction mixture was stirred at room temperature overnight, and then evaporated to dryness. The crude product (0.7 g) was used directly in step b.

b. Preparation of N-succinoyl-α-aminomyristic acid methyl ester

α-Aminomyristic acid methyl ester,HCl (0.7 g) was dissolved in chloroform (25 ml, Merck). Triethylamine (0.35 ml, Fluka) was added, followed by succinic anhydride (0.3 g, Fluka). The reaction mixture was stirred at room temperature for 2 hours, concentrated to dryness, and the residue recrystallized from ethyl acetate/petroleum ether (1/1). Yield: 0.8 g.

c. Preparation of N-(succinimidylsuccinoyl)-α-aminomyristic acid methyl ester

N-succinoyl-α-aminomyristic acid methyl ester (0.8 g) was dissolved in dry DMF (10 ml, Merck, dried over 4 Å molecular sieve). Dry pyridine (80 μl, Merck), and di(N-succinimidyl)carbonate (1.8 g, Fluka) were added, and the reaction mixture was stirred overnight at room temperature. The evaporation residue was purified by flash chromatography on silica gel 60 (Merck), and recrystallized from 2-propanol/petroleum ether (1/1). Yield of N-(succinimidylsuccinoyl)-α-aminomyristic acid methyl ester: 0.13 g, m.p. 64–66° C.

d. Reaction of (A1,B1)-diBoc human insulin with N-(succinimidylsuccinoyl)-α-aminomyristic acid methyl ester

The reaction was carried out as in Example 20 b., but using N-(succinimidylsuccinoyl)-α-aminomyristic acid methyl ester (16 mg) instead of N-(succinimidylsuberoyl)-D-thyroxine. After removal of the trifluoroacetic acid in vacuo, the evaporation residue was treated with 0.1M sodium hydroxide at 0° C. to saponify the methyl ester. When the saponification was judged to be complete by reversed phase HPLC, the pH value in the solution was adjusted to 3, and the solution was lyophilized. After purification by preparative reversed phase HPLC and desalting on a PD-10 column, the yield of $N^{\epsilon B29}$-(2-succinylamido) myristic acid human insulin was 39 mg.

The product of this example is thus human insulin wherein the ε-amino group of Lys^{B29} has a substituent of the following structure: $CH_3(CH_2)_{11}CH(COOH)NHCOCH_2C{:}H_2CO$—.

Molecular mass of the product found by MS: 6130, theory: 6133.

Example 22

Synthesis of $N^{\epsilon B29}$-octyloxycarbonyl Human Insulin

The synthesis was carried out as in Example 20 b., but using n-octyloxycarbonyl N-hydroxysuccinimide (9 mg, prepared from n-octyl chloroformate (Aldrich) and N-hydroxysuccinimide), instead of N-(succinimidylsuberoyl)-D-thyroxine. The yield of $N^{\epsilon B29}$-octyloxycarbonyl human insulin was 86 mg.

The product of this example is thus human insulin wherein the ε-amino group of Lys^{B29} has a substituent of the following structure: $CH_3(CH_2)_7OCO$—.

Molecular mass of the product found by MS: 5960, theory: 5964.

Example 23

Synthesis of $NE^{\epsilon B29}$-(2-succinylamido)palmitic acid Human Insulin

a. Preparation of N-(succinimidylsuccinoyl)-α-amino palmitic acid methyl ester

This compound was prepared as described in Example 21 a.–c., using α-amino palmitic acid instead of α-amino myristic acid.

b. Reaction of (A1,B1)-diBoc Human Insulin with N-(succinimidylsuccinoyl)-α-aminopalmitictic acid methyl ester

The reaction was carried out as in Example 21 d., but using N-(succinimidylsuccinoyl)-α-aminopalmitic acid methyl ester instead of N-(succinimidylsuccinoyl)-α-aminopalmitic acid methyl ester to give $N^{\epsilon B29}$-(2-succinylamido)palmitic acid human insulin.

The product of this example is thus human insulin wherein the ε-amino group of Lys^{B29} has a substituent of the following structure: $CH_3(CH_2)_{13}CH(COOH)NHCOCH_2CH_2CO$—.

Example 24

Synthesis of $N^{\epsilon B29}$-(2-succinylamidoethyloxy) palmitic acid Human Insulin

a. Preparation of N-(succinimidylsuccinoyl)-2-aminoethyloxy palmitic acid methyl ester

This compound was prepared as described in Example 21 a.–c. but using 2-aminoethyloxy palmitic acid (synthesized by the general procedure described by R. TenBrink, *J. Org. Chem.* 52 (1987) 418–422 instead of α-amino myristic acid.
b. Reaction of (A1,B1)-diBoc human insulin with N-(succinimidylsuccinoyl)-2-aminoethyloxypalmitictic acid methyl ester

The reaction was carried out as in Example 21 d., but using N-(succinimidylsuccinoyl)-2-aminoethyloxypalmitic acid methyl ester instead of N-(succinimidylsuccinoyl)-α-aminomyristic acid methyl ester to give $N^{\epsilon B29}$-(2-succinylamidoethyloxy)palmitic acid human insulin.

The product of this example is thus human insulin wherein the ε-amino group of Lys^{B29} has a substituent of the following structure: $CH_3(CH_2)_{13}CH(COOH)NHCH_2CH_2OCOCH_2CH_2CO$—.

Example 25

Synthesis of $N^{\epsilon B29}$-lithocholoyl-α-glutamyl des (B30) Human Insulin

The synthesis was carried out as in Example 13 using N-lithocholoyl-L-glutamic acid α-N-hydroxysuccinimide ester, γ-tert-butyl ester instead of decanoic acid N-hydroxysuccinimide ester.

The product of this example is thus des(B30) human insulin wherein the ε-amino group of Lys^{B29} has a substituent of the following structure: lithocholoyl-NHCH(CH_2CH_2COOH)CO—.

Molecular mass of the product found by MS: 6194, theory: 6193.

Example 26

Synthesis of $N^{\epsilon B29}$ -3,3',5,5'-tetraiodothyroacetyl Human Insulin

The synthesis was carried out as in Example 10 using 3,3',5,5'-tetraiodothyroacetic acid N-hydroxysuccinimide ester, instead of decanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 6536, theory: 6538.

Example 27

Synthesis of $N^{\epsilon B29}$-L-thyroxyl Human Insulin

The synthesis was carried out as in Example 10 using Boc-L-thyroxine N-hydroxysuccinimide ester, instead of decanoic acid N-hydroxysuccinimide ester.

Molecular mass of the product found by MS: 6572, theory: 6567.

Example 28

A Pharmaceutical Composition Comprising 600 nmol/ml of $N^{\epsilon B29}$-decanoyl des(B30) Human Insulin, 1/3Zn^{2+} in Solution

$N^{\epsilon B29}$-decanoyl des(B30) human insulin (1.2 μmol) was dissolved in water (0.8 ml) and the pH value was adjusted to 7.5 by addition of 0.2 M sodium hydroxide. 0.01 M zinc acetate (60 μl) and a solution containing 0.75% of phenol and 4% of glycerol (0.8 ml) was added. The pH value of the solution was adjusted to 7.5 using 0.2 M sodium hydroxide and the volume of the solution was adjusted to 2 ml with water.

The resulting solution was sterilized by filtration and transferred aseptically to a cartridge or a vial.

Example 29

A Pharmaceutical Composition Comprising 600 nmol/ml of $N^{\epsilon B29}$-decanoyl Human Insulin, ½Zn^{2+} in Solution

1.2 μmol of the title compound was dissolved in water (0.8 ml) and the pH value was adjusted to 7.5 by addition of 0.2 M sodium hydroxide. A solution containing 0.75% of phenol and 1.75% of sodium chloride (0.8 ml) was added. The pH value of the solution was adjusted to 7.5 using 0.2 M sodium hydroxide and the volume of the solution was adjusted to 2 ml with water.

The resulting solution was sterilized by filtration and transferred aseptically to a cartridge or a vial.

Example 30

A Pharmaceutical Composition Comprising 600 nmol/ml of $N^{\epsilon B29}$-lithocholoyl Human Insulin in Solution

1.2 μmol of the title compound was suspended in water (0.8 ml) and dissolved by adjusting the pH value of the solution to 8.5 using 0.2 M sodium hydroxide. To the solution was then added 0.8 ml of a stock solution containing 0.75% cresol and 4% glycerol in water. Finally, the pH value was again adjusted to 8.5 and the volume of the solution was adjusted to 2 ml with water.

The resulting solution was sterilized by filtration and transferred aseptically to a cartridge or a vial.

Example 31

A Pharmaceutical Composition Comprising a Solution of 600 nmol/ml of $N^{\epsilon B29}$-hexadecanoyl Human Insulin ⅓ Zinc Ion per Insulin Monomer, 16 mM m-cresol, 16 mM phenol, 1.6% glycerol, 10 mM sodium chloride and 7 mM sodium phosphate

1.2 μmol of $N^{\epsilon B29}$-hexadecanoyl human insulin was dissolved in water (0.5 ml) by addition of 0.2 M sodium hydroxide to pH 8.0 and 40 μl of 0.01 M zinc acetate was added. To the solution was further added 100 μl of 0.32 M phenol, 200 μl of 0.16 M m-cresol, 800 μl of 4% glycerol, 33.3 μl of 0.6 M sodium chloride, and 140 μl of 0.1 M sodium phosphate (pH 7.5). The pH value of the solution was adjusted to 7.5 with 0.1 M hydrochloric acid and the volume adjusted to 2 ml with water.

Example 32

Solubility of Various Compositions Comprising $N^{\epsilon B29}$-tetradecanoyl des(B30) Human Insulin and $N^{\epsilon B29}$-hexadecanoyl Human Insulin

The solubility of $N^{\epsilon B29}$-tetradecanoyl des(B30) human insulin and $N^{\epsilon B29}$-hexadecanoyl human insulin in different compositions was tested. The compositions were prepared as described in Example 31 with the necessary adjustment of the amount of the components. Zinc acetate was either left out or an amount corresponding to ⅓ Zn^{2+} per insulin monomer was used. Sodium chloride was used in amounts which resulted in a final concentration of 5, 25, 50, 75, 100 or 150 mM of sodium chloride. Zinc-free insulin was added to give a final amount in the composition of 1000 nmol/ml. In some cases a precipitate formed. The resulting solutions and suspensions were kept at 4° C. for a week and the concentration of insulin in solution in each composition was

then measured by high performance size exclusion chromatography relative to a standard of human insulin (column: Waters ProteinPak 250×8 mm; eluent: 2.5 M acetic acid, 4 mM arginine: 20% acetonitrile; flow rate: 1 ml/min; injection volume: 40 μl; detection: UV absorbance at 276 nm). The results, in nmol/ml, are given in the table below:

Solubility of insulins (nmol/ml) in 16 mM phenol, 16 mM m-cresol, 1.6% glycerol, 7 mM sodium phosphate, and pH 7.5, varying zinc acetate and sodium chloride concentrations at 4° C.	Sodium chloride 5 mM	25 mM	50 mM	75 mM	100 mM	150 mM
$N^{\epsilon B29}$-tetradecanoyl des(B30) human insulin, zinc-free.	82	115	54	77	74	84
$N^{\epsilon B29}$-tetradecanoyl des(B30) human insulin, 1/3 Zn^{2+} per insulin monomer.	>950	>950	>950	>950	>950	485
$N^{\epsilon B29}$-hexadecanoyl human insulin, zinc-free.	>890	>950	283	106	45	29
$N^{\epsilon B29}$-hexadecanoyl human insulin, 1/3 Zn^{2+} per insulin monomer.	>950	>950	>950	>950	920	620

In conclusion it appears that the solubility of the acylated insulins is increased by the addition of zinc. This is contrary to published data on human, porcine and bovine insulin (J Brange: Galenics of Insulin, page 19, Springer Verlag (1987); J Markussen et al. *Protein Engineering* 1 (1987) 205–213).

Example 33

Preparative Crystallization of Zinc-Free $N^{\epsilon B29}$-tetradecanoyl des(B30) Human Insulin

10 g of $N^{\epsilon B29}$-tetradecanoyl des(B30) human insulin was dissolved in 120 ml of 0.02 M NH_4Cl buffer adjusted to pH 9.0 with NH_3 in ethanol/water (1:4, v/v). Gentle stirring was maintained throughout the crystallization. Crystallization was initiated at 23° C. by addition of 20 ml of 2.5 M NaCl dissolved in ethanol/water (1:4, v/v). A slight turbidity appeared in the solution. Further, 20 ml of 2.5 M sodium chloride dissolved in ethanol/water (1:4, v/v) was added at a constant rate of 5 ml/h, which caused the crystallization to proceed slowly. In order to decrease the solubility of the insulin, the pH value was then adjusted to 7.5 using 1 N hydrochloric acid. Finally, the temperature was lowered to 4° C. and the stirring continued overnight. The crystals were collected by filtration, washed twice with 25 ml of 0.2 M NaCl in ethanol/water (1:4, v/v), sucked dry and lyophilized.

The weight of the wet filter cake was 19.33 g.

The weight of lyophilized filter cake was 9.71 g.

Example 34

Synthesis of Lys^{B29}(N^{ϵ}-[N^{α}-tetradecanoyl-Glu-Gly-1) des(B30) Human Insulin

500 mg of (A1,B1)-diBoc human insulin was dissolved in a mixture of 186 μl of 4-methylmorpholine and 3814 μl of DMSO. The reaction was initiated by addition of 144 mg of tetradecanoyl-Glu(γ-OtBu)-Gly-OSu dissolved in 1000 μl of DMF. The reaction conducted at 15° C. and it was stopped after 4.5 hours by addition of 100 ml of acetone. The reaction product precipitated by addition of a few drops of concentrated HCl was subsequently isolated by centrifugation. The precipitate was then suspended in 100 ml of acetone, isolated by centrifugation and dried in vacuum. 637 mg of material was obtained.

The Boc protecting groups were eliminated by addition of 5 ml of TFA. The dissolved material was incubated for 30 minutes and then precipitated by addition of 100 ml of acetone and a few drops of concentrated HCl. The precipitate was then suspended in 100 ml acetone and isolated by centrifugation. The precipitated material was dissolved in 200 ml of 25% ethanol at pH 8 by addition of NH_4OH and purified by reversed phase HPLC. The dissolved material was applied to a column (5 cm diameter, 30 cm high) packed with octadecyldimethylsilyl-substituted silica particles (mean particle size 15 μm, pore size 100 Å) and equilibrated with 0.02 M Bis-Tris, 30% ethanol adjusted to pH 7.3 with hydrochloric acid at a temperature of 40° C. The elution was performed using mixtures of 70% ethanol in water and Bis-Tris buffer. The flow was 2 l/h. The insulin was eluted by increasing the ethanol content from 30% to 50% and the effluent was monitored by its UV absorbance at 280 nm. The appropriate fraction was diluted to 20% ethanol adjusted to pH 4.5 and frozen at −20° C. The precipitated material was isolated after equilibration of the sample at 1° C. and subsequent centrifugation at the same temperature. The precipitate was dried in vacuum. Thus 292 mg of the title compound was obtained at a purity of 95.5%.

Molecular mass, found by MS: 6102±6, theory: 6103.

The lipophilicity of the title compound, relative to human insulin, k'_{rel}=20. The determination was carried out as described on page 23 of the description.

The disappearance half-life, $T_{50\%}$, of the title compound after subcutaneous injection in pigs was found to be 11.9 hours. The determination was carried out as described on page 24 of the description using a composition similar to those described in Table 2 on page 26 of the description.

Example 35

Synthesis of Lys^{B29}(N^{ϵ}-tetradecanoyl-Glu-) des (B30) Human Insulin

500 mg of (A1,B1)-diBoc human insulin was dissolved in a mixture of 186 μl of 4-methylmorpholine and 3814 μl of DMSO. The reaction was initiated by addition of 85 mg of N^{α}-tetradecanoyl-Glu(OtBu)-OSu dissolved in 1000 μl of DMF. The reaction was conducted at 15° C. and it was stopped after 4.5 hours. The remaining process steps were performed as described in Example 34. The intermediate product was isolated and the protection groups were removed by TFA before purification by RP-HPLC and final isolation by precipitation and vacuum drying.

Thus 356 mg of the title compound was obtained at a purity of 94.1%. Molecular mass, found by MS: 6053±6, theory: 6046.

The lipophilicity of the title compound, relative to human insulin, k'_{rel}=24. The determination was carried out as described on page 23 of the description.

The disappearance half-life, $T_{50\%}$, of the title compound after subcutaneous injection in pigs was found to be 8.8 hours. The determination was carried out as described on page 24 of the description using a composition similar to those described in Table 2 on page 26 of the description.

Example 36

Synthesis of Lys^{B29}(N^{ϵ}-[N^{α}-tetradecanoyl-Glu(−)—OH]) Human Insulin

400 mg of (A1,B1)-diBoc human insulin was dissolved in a mixture of 232 μl of ethyldiisopropylamine, 1880 μl of

DMSO and 2088 μl of 1-methyl-2-pyrrolidone. The reaction was initiated by addition of 138 mg of N^{α}-tetradecanoyl-Glu(OSu)-OtBu dissolved in 800 μl of 1-methyl-2-pyrrolidone. The reaction was conducted at 15° C. and it was stopped after 4.5 hours. The remaining process steps were performed as described in Example 34. The protection groups were removed from the intermediate product by TFA before purification by RP-HPLC and final isolation by precipitation and vacuum drying.

Thus 222 mg of the title compound was obtained at a purity of 95.5%. Molecular mass, found by MS: 6150±6, theory: 6147.

The lipophilicity of the title compound, relative to human insulin, k'_{rel}=21. The determination was carried out as described on page 23 of the description.

The disappearance half-life, $T_{50\%}$, of the title compound after subcutaneous injection in pigs was found to be 8.0 hours. The determination was carried out as described on page 24 of the description using a composition similar to the one described in the present Example 31.

Example 37

Synthesis of Lys^{B29}(N^{ϵ}-[N^{α}-hexadecanoyl-Glu(-)—OH]) Human Insulin

400 mg of (A1,B1)-diBoc human insulin was dissolved in a mixture of 232 μl of ethyldiisopropylamine, 880 μl of DMSO and 2088 μl of 1-methyl-2-pyrrolidone. The reaction was initiated by addition of 73 mg of N^{α}-hexadecanoyl-Glu (OSu)-OtBu dissolved in 800 μl of DMF. The reaction was conducted at 150° C. and it was stopped after 4.5 hours. The remaining process steps were performed as described in Example 34. 476 mg of intermediate product was obtained. The protection groups were removed from the intermediate product by TFA before purification by RP-HPLC and final isolation by precipitation and vacuum drying.

Thus 222 mg of the title compound was obtained at a purity of 81.2%. Molecular mass, found by MS: 6179±6, theory: 6175.

The lipophilicity of the title compound, relative to human insulin, k'_{rel}=67. The determination was carried out as described on page 23 of the description.

The disappearance half-life, $T_{50\%}$, of the title compound after subcutaneous injection in pigs was found to be 13.0 hours. The determination was carried out as described on page 24 of the description using a composition similar to the one described in the present Example 31.

Example 38

Synthesis of Lys^{B29}(N^{ϵ}-[N^{α}-octadecanoyl-Glu(-)—OH]) des(B30) Human Insulin

400 mg of (A1,B1)-diBoc des(B30) human insulin was dissolved in a mixture of 232 μl of ethyldiisopropylamine, 3000 μl of DMSO and 268 μl of dimetylformamide. The reaction was initiated by addition of 114 mg N^{α}-octadecanoyl-Glu(OSu)-OtBu dissolved in 500 μl of DMF. The reaction was conducted at 15° C. and it was stopped after 4.5 hours. The remaining process steps were performed as described in Example 34. 420 mg of intermediate product was obtained. The protection groups were removed from the intermediate product by TFA before purification by RP-HPLC and final isolation by precipitation and vacuum drying.

Thus 169 mg of the title compound was obtained at a purity of 98.3%. Molecular mass, found by MS: 6103±5, theory: 6102.

The lipophilicity of the title compound, relative to human insulin, k'_{rel}=185. The determination was carried out as described on page 23 of the description.

The disappearance half-life, $T_{50\%}$, of the title compound after subcutaneous injection in pigs was found to be 9.7 hours. The determination was carried out as described on page 24 of the description using a composition similar to the one described in the present Example 31.

Example 39

Synthesis of Lys^{B29}(N^{ϵ}-[N^{α}-tetradecanoyl-Glu(-)—OH]) des(B30) Human Insulin

400 mg of (A1,B1)-diBoc des(B30) human insulin was dissolved in a mixture of 232 μl of ethyldiisopropylamine and 3000 μl of DMSO. The reaction was initiated by addition of 138 mg of N^{α}-tetradecanoyl-Glu(OSu)-OtBu dissolved in 768 μl of DMF. The reaction was conducted at 15° C. and it was stopped after 4.5 hours. The remaining process steps were performed as described in Example 34. 505 mg of intermediate product was obtained. The protection groups of the intermediate product were removed by TFA before purification by RP-HPLC and final isolation by precipitation and vacuum drying.

Thus 237 mg of the title compound was obtained at a purity of 96.7%. Molecular mass, found by MS: 6053±6, theory: 6046.

The lipophilicity of the title compound, relative to human insulin, k'_{rel}=21. The determination was carried out as described on page 23 of the description.

The disappearance half-life, $T_{50\%}$, of the title compound after subcutaneous injection in pigs was found to be 12.8 hours. The determination was carried out as described on page 24 of the description using a composition similar to the one described in the present Example 31.

Example 40

Synthesis of Lys^{B29}(N^{ϵ}-[N^{α}-hexadecanoyl-Glu(-)—OH]) des(B30) Human Insulin

400 mg of (A1,B1)-diBoc des(B30) human insulin was dissolved in a mixture of 232 μl of ethyldiisopropylamine, 3000 μl of DMSO and 400 μl of dimetylformamide. The reaction was initiated by addition of 73 mg of N^{α}-hexadecanoyl-Glu(OSu)-OtBu dissolved in 400 μl of DMF. The reaction was conducted at 15° C. and it was stopped after 4.5 hours. The remaining process steps were performed as described in Example 34. The protection groups of the intermediate product were removed by TFA before purification by RP-HPLC and final isolation by precipitation and vacuum drying.

Thus 153 mg of the title compound was obtained at a purity of 95.2%. Molecular Mass, found by MS: 6073±6, theory: 6074.

The lipophilicity of the title compound, relative to human insulin, k'_{rel}=67. The determination was carried out as described on page 23 of the description.

The disappearance half-life, $T_{50\%}$, of the title compound after subcutaneous injection in pigs was found to be 18.0 hours. The determination was carried out as described on page 24 of the description using a composition similar to the one described in the present Example 31.

Example 41

Synthesis of Lys^{B29}(N^{ϵ}-[N^{α}-lithocholyl-Glu(-)—OH]) des(B30) Human Insulin

400 mg of (A1,B1)-diBoc des(B30) human insulin was dissolved in a mixture of 148 μl 4-methylmorpholine and

3452 μl of DMSO. The reaction was initiated by addition of 132 mg of N^{α}-lithocholoyl-Glu(OSu)-OtBu dissolved in 400 μl of DMF. The reaction was conducted at 15° C. and it was stopped after 4.5 hours. The remaining process steps were performed as described in Example 34. 493 mg of intermediate product was obtained. The protection groups of the intermediate product were removed by TFA before purification by RP-HPLC and final isolation by precipitation and vacuum drying.

Thus 209 mg of the title compound was obtained at a purity of 97.4%. Molecular Mass, found by MS: 6185±10, theory: 6194.

Example 42

$Lys^{B29}(N^{\epsilon}$-N^{α}-tetradecanoyl Aad(-)—OH]) des (B30) Human Insulin

Aad is 5-aminohexadioic acid. 347 mg of (A1,B1)-diBoc des(B30) human insulin was dissolved in a mixture of 129 μl of 4-methylmorpholine and 2645 μl of DMSO. The reaction was initiated by addition of 58 mg of N^{α}-tetradecanoyl-Aad(OSu)-OtBu dissolved in 694 μl of DMF. The activated ester was prepared in analogy with chemistry well-known from as aspartic acid derivatisation (L. Benoiton: Can.J.Chem.40,570–72,1962, R. Roeske: J.Org.Chem 28 1251–93 (1963)). The reaction was conducted at 15° C. and it was stopped after 4.5 hours. The remaining process steps were performed as described in Example 34. The protection groups of the intermediate product were removed by TFA before purification by RP-HPLC and final isolation by precipitation and vacuum drying.

Thus 149 mg of the title compound was obtained at a purity of 97.9%. Molecular Mass, found by MS: 6061±2, theory: 6060.

The lipophilicity of the title compound, relative to human insulin, k'_{rel}=21. The determination was carried out as described on page 23 of the description.

The disappearance half-life, $T_{50\%}$, of the title compound after subcutaneous injection in pigs was found to be 16.1 hours. The determination was carried out as described on page 24 of the description using a composition similar to the one described in the present Example 31.

Example 43

Synthesis of $Lys^{B29}(N^{\epsilon}$-[N^{α}-tetradecanoyl-γ-carboxy-Glu-]) des(B30) Human Insulin

400 mg of (A1,B1)-diBoc des(B30) human insulin was dissolved in a mixture of 190 μl of triethylamine and 3000 μl of DMSO. The reaction was initiated by addition of 83 mg of γ-carboxy Glu N-tetradecansyre γ,γ'-di(OtBu) α-(OSu) (i.e. $(tBuOCO)_2CHCH_2$—CH(COOSu)-NH—$CO(CH_2)_{12}CH_3$) dissolved in 800 μl of DMF. The reaction was conducted at 15° C. and it was stopped after 4.5 hours. The remaining process steps were performed as described in Example 34. The protection groups of the intermediate product were removed by TFA before purification by RP-HPLC and final isolation by precipitation and vacuum drying.

63 mg of the title compound were obtained. Molecular Mass, found by MS: 6090 ±3, theory: 6091.

The lipophilicity of the title compound, relative to human insulin, k'_{rel}=10. The determination was carried out as described on page 23 of the description.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 49

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu
1 5 10 15

Glu Asn Tyr Cys Xaa
20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Xaa Val Xaa Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr

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1 5 10 15

Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Xaa
20 25 30

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGGCTAAGAG ATTCGTTGAC CAACACTTGT GCGGTTCTCA CTTGGTTGAA GCTTTGTACT 60

TGGTTTGTGG TGAAAGAGGT TTCTTCTACA CTCCAAAGTC TGACGACGCT 110

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 100 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTGCGGGCTG CGTCTAAGCA CAGTAGTTTT CCAATTGGTA CAAAGAACAG ATAGAAGTAC 60

AACATTGTTC AACGATACCC TTAGCGTCGT CAGACTTTGG 100

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTCGCCATGG CTAAGAGATT CGTTG 25

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTGCTCTAGA GCCTGCGGGC TGCGTCT 27

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TGGCTAAGAG ATTCGTTACT CAACACTTGT GCGGTTCTCA CTTGGTTGAA GCTTTGTACT 60

TGGTTTGTGG TGAAAGAGGT TTCTTCTACA CTCCAAAGTC TGACGACGCT 110

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTCGCCATGG CTAAGAGATT CGTTA 25

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 100 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTGCGGGCTG CGTCTAACCA CAGTAGTTTT CCAATTGGTA CAAAGAACAG ATAGAAGTAC 60

AACATTGTTC AACGATACCC TTAGCGTCGT CAGACTTTGG 100

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ACGTACGTTC TAGAGCCTGC GGGCTGC 27

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 78 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CACTTGGTTG AAGCTTTGTA CTTGGTTTGT GGTGAAAGAG GTTTCTTCTA CACTCCAAAG 60

ACTAGAGGTA TCGTTGAA 78

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

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CAC 63

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCTAACGTCG CCATGGCTAA GAGAGAAGAA GCTGAAGCGA AGCTGAAAGA TTCGTTAACC 60

AACAC 65

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 415 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACTATCAA TTTCATACAC 60

AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC 112
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
1 5 10

GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG 160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
15 20 25

ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC 208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
30 35 40

GTC GCC ATG GCT AAG AGA TTC GTT AAC CAA CAC TTG TGC GGT TCT CAC 256
Val Ala Met Ala Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His
45 50 55

TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC 304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
60 65 70 75

ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT 352
Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr
80 85 90

TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT AAC TAGACGCAGC 401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
95 100

CCGCAGGCTC TAGA 415

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 104 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

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Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 1               5                  10                  15

Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
            20                  25                  30

Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
        35                  40                  45

Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
    50                  55                  60

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
65                  70                  75                  80

Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
                85                  90                  95

Tyr Gln Leu Glu Asn Tyr Cys Asn
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(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 415 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

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TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG     60

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GACCCGGGTT GGTCAGTGAC CGCTACTTAG TAGACAACTC TAAGGCCTTC TCAGAGACTA    180

GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AATTGGTTGT    240

GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA    300

GATGTGAGGT TTCAGACTGC TGCGATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC    360

AAGAAACATG GTTAACCTTT TGATGACATT GATCTGCGTC GGGCGTCCGA GATCT         415
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(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 523 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 80..499

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

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                     Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
                       1               5                  10

TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA      160
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu
            15                  20                  25
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GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT      208
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp
         30                  35                  40

TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA      256
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr
     45                  50                  55

AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT      304
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala
 60                  65                  70                  75

AAA GAA GAA GGG GTA TCT TTG GAT AAG AGA GAA GTT AAC CAA CAC TTG      352
Lys Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Asn Gln His Leu
                 80                  85                  90

TGC GGT TCT CAC TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA      400
Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg
             95                  100                 105

GGT TTC TTC TAC ACT GAA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA      448
Gly Phe Phe Tyr Thr Glu Lys Ser Asp Asp Ala Lys Gly Ile Val Glu
         110                 115                 120

CAA TGT TGT ACT TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT      496
Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys
     125                 130                 135

AAC TAGACGCAGC CCGCAGGCTC TAGA                                      523
Asn
140
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 140 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
  1               5                  10                  15

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
             20                  25                  30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
         35                  40                  45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
     50                  55                  60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
 65                  70                  75                  80

Ser Leu Asp Lys Arg Glu Val Asn Gln His Leu Cys Gly Ser His Leu
                 85                  90                  95

Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr
             100                 105                 110

Glu Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser
         115                 120                 125

Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
     130                 135                 140
```

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 523 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG     60

TTATATTTGC TAATTTTCTT ACTCTAAAGG AAGTTAAAAA TGACGTCAAA ATAAGCGTCG    120

TAGGAGGCGT AATCGACGAG GTCAGTTGTG ATGTTGTCTT CTACTTTGCC GTGTTTAAGG    180

CCGACTTCGA CAGTAGCCAA TGAGTCTAAA TCTTCCCCTA AAGCTACAAC GACAAAACGG    240

TAAAAGGTTG TCGTGTTTAT TGCCCAATAA CAAATATTTA TGATGATAAC GGTCGTAACG    300

ACGATTTCTT CTTCCCCATA GAAACCTATT CTCTCTTCAA TTGGTTGTGA ACACGCCAAG    360

AGTGAACCAA CTTCGAAACA TGAACCAAAC ACCACTTTCT CCAAAGAAGA TGTGACTTTT    420

CAGACTGCTG CGATTCCCAT AGCAACTTGT TACAACATGA AGATAGACAA GAAACATGGT    480

TAACCTTTTG ATGACATTGA TCTGCGTCGG GCGTCCGAGA TCT                      523
```

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 415 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACTATCAA TTTCATACAC     60

AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC     112
                     Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
                       1               5                  10

GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG      160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
            15                  20                  25

ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC      208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
        30                  35                  40

GTC GCC ATG GCT AAG AGA TTC GTT GAC CAA CAC TTG TGC GGT TCT CAC      256
Val Ala Met Ala Lys Arg Phe Val Asp Gln His Leu Cys Gly Ser His
    45                  50                  55

TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC      304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
60                  65                  70                  75

ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT      352
Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr
                80                  85                  90

TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GCT TAGACGCAGC       401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Ala
            95                  100

CCGCAGGCTC TAGA                                                     415
```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 104 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 1               5                  10                  15

Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
            20                  25                  30

Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
        35                  40                  45

Arg Phe Val Asp Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
    50                  55                  60

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
65                  70                  75                  80

Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
                85                  90                  95

Tyr Gln Leu Glu Asn Tyr Cys Ala
            100
```

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 415 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG     60

TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC    120

GACCCGGGTT GGTCAGTGAC CGCTACTTAG TAGACAACTC TAAGGCCTTC TCAGAGACTA    180

GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AACTGGTTGT    240

GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA    300

GATGTGAGGT TTCAGACTGC TGCGATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC    360

AAGAAACATG GTTAACCTTT TGATGACACG AATCTGCGTC GGGCGTCCGA GATCT         415
```

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 415 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACTATCAA TTTCATACAC     60

AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC     112
                     Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
                       1               5                  10

GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG      160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
            15                  20                  25

ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC      208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
```

-continued

```
        30                  35                  40

GTC GCC ATG GCT AAG AGA TTC GTT ACT CAA CAC TTG TGC GGT TCT CAC      256
Val Ala Met Ala Lys Arg Phe Val Thr Gln His Leu Cys Gly Ser His
     45                  50                  55

TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC      304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
 60                  65                  70                  75

ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT      352
Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr
                 80                  85                  90

TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GCT TAGACGCAGC       401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Ala
             95                  100

CCGCAGGCTC TAGA                                                     415
```

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 104 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
  1               5                  10                  15

Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
            20                  25                  30

Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
        35                  40                  45

Arg Phe Val Thr Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
    50                  55                  60

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
65                  70                  75                  80

Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
                85                  90                  95

Tyr Gln Leu Glu Asn Tyr Cys Ala
            100
```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 415 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```
TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG     60

TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC    120

GACCCGGGTT GGTCAGTGAC CGCTACTTAG TAGACAACTC TAAGGCCTTC TCAGAGACTA    180

GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AATGAGTTGT    240

GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA    300

GATGTGAGGT TTCAGACTGC TGCGATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC    360

AAGAAACATG GTTAACCTTT TGATGACACG AATCTGCGTC GGGCGTCCGA GATCT         415
```

-continued

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 415 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```
ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACTATCAA TTTCATACAC      60

AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC      112
                     Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
                       1               5                  10

GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG      160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
            15                  20                  25

ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC      208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
        30                  35                  40

GTC GCC ATG GCT AAG AGA TTC GTT GAC CAA CAC TTG TGC GGT TCT CAC      256
Val Ala Met Ala Lys Arg Phe Val Asp Gln His Leu Cys Gly Ser His
    45                  50                  55

TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC      304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
60                  65                  70                  75

ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT      352
Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr
                80                  85                  90

TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GGT TAGACGCAGC      401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Gly
            95                  100

CCGCAGGCTC TAGA                                                     415
```

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 104 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

```
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
  1               5                  10                  15

Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
            20                  25                  30

Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
        35                  40                  45

Arg Phe Val Asp Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
    50                  55                  60

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
65                  70                  75                  80

Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
                85                  90                  95

Tyr Gln Leu Glu Asn Tyr Cys Gly
```

-continued

100

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 415 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

```
TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG     60

TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC    120

GACCCGGGTT GGTCAGTGAC CGCTACTTAG TAGACAACTC TAAGGCCTTC TCAGAGACTA    180

GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AACTGGTTGT    240

GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA    300

GATGTGAGGT TTCAGACTGC TGCGATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC    360

AAGAAACATG GTTAACCTTT TGATGACACC AATCTGCGTC GGGCGTCCGA GATCT         415
```

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 415 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 80..391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

```
ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACTATCAA TTTCATACAC     60

AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC     112
                     Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
                       1               5                  10

GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG      160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
            15                  20                  25

ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC      208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
        30                  35                  40

GTC GCC ATG GCT AAG AGA TTC GTT ACT CAA CAC TTG TGC GGT TCT CAC      256
Val Ala Met Ala Lys Arg Phe Val Thr Gln His Leu Cys Gly Ser His
    45                  50                  55

TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC      304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
60                  65                  70                  75

ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA CAA TGT TGT ACT      352
Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr
                80                  85                  90

TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GGT TAGACGCAGC       401
Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Gly
            95                  100

CCGCAGGCTC TAGA                                                     415
```

(2) INFORMATION FOR SEQ ID NO:30:

-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 104 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

```
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 1               5                  10                  15

Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
            20                  25                  30

Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
        35                  40                  45

Arg Phe Val Thr Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
    50                  55                  60

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ser Asp
65                  70                  75                  80

Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu
                85                  90                  95

Tyr Gln Leu Glu Asn Tyr Cys Gly
            100
```

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 415 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

```
TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG     60

TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC    120

GACCCGGGTT GGTCAGTGAC CGCTACTTAG TAGACAACTC TAAGGCCTTC TCAGAGACTA    180

GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AATGAGTTGT    240

GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA    300

GATGTGAGGT TTCAGACTGC TGCGATTCCC ATAGCAACTT GTTACAACAT GAAGATAGAC    360

AAGAAACATG GTTAACCTTT TGATGACACC AATCTGCGTC GGGCGTCCGA GATCT         415
```

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 523 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 80..499

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

```
ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACTATCAA TTTCATACAC     60

AATATAAACG ATTAAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA     112
                     Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
                       1               5                  10
```

-continued

```
TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA      160
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu
            15                  20                  25

GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT      208
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp
        30                  35                  40

TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA      256
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr
    45                  50                  55

AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT      304
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala
60                  65                  70                  75

AAA GAA GAA GGG GTA TCT TTG GAT AAG AGA TTC GTT AAC CAA CAC TTG      352
Lys Glu Glu Gly Val Ser Leu Asp Lys Arg Phe Val Asn Gln His Leu
                80                  85                  90

TGC GGT TCT CAC TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA      400
Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg
            95                  100                 105

GGT TTC TTC TAC ACT CCA AAG TCT GAC GAC GCT AAG GGT ATC GTT GAA      448
Gly Phe Phe Tyr Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu
        110                 115                 120

CAA TGT TGT ACT TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT      496
Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys
    125                 130                 135

AAC TAGACGCAGC CCGCAGGCTC TAGA                                      523
Asn
140
```

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 140 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

```
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
  1               5                  10                  15

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
            20                  25                  30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
        35                  40                  45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
    50                  55                  60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
65                  70                  75                  80

Ser Leu Asp Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu
                85                  90                  95

Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr
            100                 105                 110

Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser
        115                 120                 125

Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
    130                 135                 140
```

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

-continued

(A) LENGTH: 523 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

```
TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG     60

TTATATTTGC TAATTTTCTT ACTCTAAAGG AAGTTAAAAA TGACGTCAAA ATAAGCGTCG    120

TAGGAGGCGT AATCGACGAG GTCAGTTGTG ATGTTGTCTT CTACTTTGCC GTGTTTAAGG    180

CCGACTTCGA CAGTAGCCAA TGAGTCTAAA TCTTCCCCTA AAGCTACAAC GACAAAACGG    240

TAAAAGGTTG TCGTGTTTAT TGCCCAATAA CAAATATTTA TGATGATAAC GGTCGTAACG    300

ACGATTTCTT CTTCCCCATA GAAACCTATT CTCTAAGCAA TTGGTTGTGA ACACGCCAAG    360

AGTGAACCAA CTTCGAAACA TGAACCAAAC ACCACTTTCT CCAAAGAAGA TGTGAGGTTT    420

CAGACTGCTG CGATTCCCAT AGCAACTTGT TACAACATGA AGATAGACAA GAAACATGGT    480

TAACCTTTTG ATGACATTGA TCTGCGTCGG GCGTCCGAGA TCT                      523
```

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 409 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 80..385

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

```
ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACTATCAA TTTCATACAC     60

AATATAAACG ACCAAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC     112
                     Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
                       1               5                  10

GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG      160
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
            15                  20                  25

ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC      208
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
        30                  35                  40

GTC GCC ATG GCT AAG AGA TTC GTT AAC CAA CAC TTG TGC GGT TCT CAC      256
Val Ala Met Ala Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His
    45                  50                  55

TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC      304
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
60                  65                  70                  75

ACT CCT AAG GAA AAG AGA GGT ATC GTT GAA CAA TGT TGT ACT TCT ATC      352
Thr Pro Lys Glu Lys Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile
                80                  85                  90

TGT TCT TTG TAC CAA TTG GAA AAC TAC TGT GGT TAGACGCAGC CCGCAGGCTC    405
Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Gly
            95                  100

TAGA                                                                409
```

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

-continued

(A) LENGTH: 102 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

```
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 1               5                  10                  15

Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
            20                  25                  30

Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys
        35                  40                  45

Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
    50                  55                  60

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Glu Lys
65                  70                  75                  80

Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln
                85                  90                  95

Leu Glu Asn Tyr Cys Gly
            100
```

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 409 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

```
TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG     60

TTATATTTGC TGGTTTTCTT ACTTCCGACA AAAGAACCAA AACAGGAACT AGCCTAAGAC    120

GACCCGGGTT GGTCAGTGAC CGCTACTTAG TAGACAACTC TAAGGCCTTC TCAGAGACTA    180

GTAGCGACTT TTGTGGTGAA ACCGATTGCA GCGGTACCGA TTCTCTAAGC AATTGGTTGT    240

GAACACGCCA AGAGTGAACC AACTTCGAAA CATGAACCAA ACACCACTTT CTCCAAAGAA    300

GATGTGAGGA TTCCTTTTCT CTCCATAGCA ACTTGTTACA ACATGAAGAT AGACAAGAAA    360

CATGGTTAAC CTTTTGATGA CACCAATCTG CGTCGGGCGT CCGAGATCT                409
```

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 511 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 77..487

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

```
GAATTCCATT CAAGAATAGT TCAAACAAGA AGATTACAAA CTATCAATTT CATACACAAT     60

ATAAACGATT AAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA         109
                  Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
                    1               5                  10

TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA       157
```

-continued

```
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu
             15                  20                  25

GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT      205
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp
         30                  35                  40

TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA      253
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr
     45                  50                  55

AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT      301
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala
 60                  65                  70                  75

AAA GAA GAA GGG GTA TCC ATG GCT AAG AGA TTC GTT AAC CAA CAC TTG      349
Lys Glu Glu Gly Val Ser Met Ala Lys Arg Phe Val Asn Gln His Leu
                 80                  85                  90

TGC GGT TCC CAC TTG GTT GAA GCT TTG TAC TTG GTT TGT GGT GAA AGA      397
Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg
             95                  100                 105

GGT TTC TTC TAC ACT CCA AAG ACT AGA GGT ATC GTT GAA CAA TGT TGT      445
Gly Phe Phe Tyr Thr Pro Lys Thr Arg Gly Ile Val Glu Gln Cys Cys
         110                 115                 120

ACT TCT ATC TGT TCT TTG TAC CAA TTG GAA AAC TAC TGC AAC              487
Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
     125                 130                 135

TAGACGCAGC CCGCAGGCTC TAGA                                          511
```

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 137 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

```
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
  1               5                  10                  15

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
             20                  25                  30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
         35                  40                  45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
     50                  55                  60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
 65                  70                  75                  80

Ser Met Ala Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu
                 85                  90                  95

Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr
            100                 105                 110

Pro Lys Thr Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser
        115                 120                 125

Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
    130                 135
```

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 511 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

```
CTTAAGGTAA GTTCTTATCA AGTTTGTTCT TCTAATGTTT GATAGTTAAA GTATGTGTTA     60

TATTTGCTAA TTTTCTTACT CTAAAGGAAG TTAAAAATGA CGTCAAAATA AGCGTCGTAG    120

GAGGCGTAAT CGACGAGGTC AGTTGTGATG TTGTCTTCTA CTTTGCCGTG TTTAAGGCCG    180

ACTTCGACAG TAGCCAATGA GTCTAAATCT TCCCCTAAAG CTACAACGAC AAAACGGTAA    240

AAGGTTGTCG TGTTTATTGC CCAATAACAA ATATTTATGA TGATAACGGT CGTAACGACG    300

ATTTCTTCTT CCCCATAGGT ACCGATTCTC TAAGCAATTG GTTGTGAACA CGCCAAGGGT    360

GAACCAACTT CGAAACATGA ACCAAACACC ACTTTCTCCA AAGAAGATGT GAGGTTTCTG    420

ATCTCCATAG CAACTTGTTA CAACATGAAG ATAGACAAGA AACATGGTTA ACCTTTTGAT    480

GACGTTGATC TGCGTCGGGC GTCCGAGATC T                                    511
```

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 523 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 80..499

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

```
ATCGAATTCC ATTCAAGAAT AGTTCAAACA AGAAGATTAC AAACTATCAA TTTCATACAC     60

AATATAAACG ATTAAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA      112
                     Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
                       1               5                  10

TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA      160
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu
            15                  20                  25

GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT      208
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp
        30                  35                  40

TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA      256
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr
    45                  50                  55

AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT      304
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala
60                  65                  70                  75

AAA GAA GAA GGG GTA TCC ATG GCT AAG AGA TTC GTT AAC CAA CAC TTG      352
Lys Glu Glu Gly Val Ser Met Ala Lys Arg Phe Val Asn Gln His Leu
                80                  85                  90

TGC GGT TCC CAC TTG GTT GAA GCT TTG TAC TTG GTT TGC GGT GAA AGA      400
Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg
            95                  100                 105

GGT TTC TTC TAC ACT CCT AAG TCT GAC GAT GCT AAG GGT ATT GTC GAG      448
Gly Phe Phe Tyr Thr Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu
        110                 115                 120

CAA TGC TGT ACC TCC ATC TGC TCC TTG TAC CAA TTG GAA AAC TAC TGC      496
Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys
    125                 130                 135

AAC TAGACGCAGC CCGCAGGCTC TAGA                                      523
Asn
```

-continued

140

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 140 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

```
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
  1               5                  10                  15

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
            20                  25                  30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
        35                  40                  45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
    50                  55                  60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
65                  70                  75                  80

Ser Met Ala Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu
                85                  90                  95

Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr
            100                 105                 110

Pro Lys Ser Asp Asp Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser
        115                 120                 125

Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
    130                 135                 140
```

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 523 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

```
TAGCTTAAGG TAAGTTCTTA TCAAGTTTGT TCTTCTAATG TTTGATAGTT AAAGTATGTG     60

TTATATTTGC TAATTTTCTT ACTCTAAAGG AAGTTAAAAA TGACGTCAAA ATAAGCGTCG    120

TAGGAGGCGT AATCGACGAG GTCAGTTGTG ATGTTGTCTT CTACTTTGCC GTGTTTAAGG    180

CCGACTTCGA CAGTAGCCAA TGAGTCTAAA TCTTCCCCTA AAGCTACAAC GACAAAACGG    240

TAAAAGGTTG TCGTGTTTAT TGCCCAATAA CAAATATTTA TGATGATAAC GGTCGTAACG    300

ACGATTTCTT CTTCCCCATA GGTACCGATT CTCTAAGCAA TTGGTTGTGA ACACGCCAAG    360

GGTGAACCAA CTTCGAAACA TGAACCAAAC GCCACTTTCT CCAAAGAAGA TGTGAGGATT    420

CAGACTGCTA CGATTCCCAT AACAGCTCGT TACGACATGG AGGTAGACGA GGAACATGGT    480

TAACCTTTTG ATGACGTTGA TCTGCGTCGG GCGTCCGAGA TCT                      523
```

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 535 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 77..511

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

```
GAATTCCATT CAAGAATAGT TCAAACAAGA AGATTACAAA CTATCAATTT CATACACAAT      60

ATAAACGATT AAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA          109
                  Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
                    1               5                  10

TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA       157
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu
            15                  20                  25

GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT       205
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp
        30                  35                  40

TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA       253
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr
    45                  50                  55

AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT       301
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala
60                  65                  70                  75

AAA GAA GAA GGG GTA TCC ATG GCT AAG AGA GAA GAA GCT GAA GCT GAA       349
Lys Glu Glu Gly Val Ser Met Ala Lys Arg Glu Glu Ala Glu Ala Glu
                80                  85                  90

GCT AGA TTC GTT AAC CAA CAC TTG TGC GGT TCC CAC TTG GTT GAA GCT       397
Ala Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala
            95                  100                 105

TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC ACT CCA AAG ACT       445
Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr
        110                 115                 120

AGA GGT ATC GTT GAA CAA TGT TGT ACT TCT ATC TGT TCT TTG TAC CAA       493
Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln
    125                 130                 135

TTG GAA AAC TAC TGC AAC TAGACGCAGC CCGCAGGCTC TAGA                    535
Leu Glu Asn Tyr Cys Asn
140                 145
```

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 145 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

```
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
  1               5                  10                  15

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
            20                  25                  30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
        35                  40                  45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
    50                  55                  60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
65                  70                  75                  80

Ser Met Ala Lys Arg Glu Glu Ala Glu Ala Glu Ala Arg Phe Val Asn
                85                  90                  95
```

-continued

```
Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys
            100                 105                 110

Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr Arg Gly Ile Val Glu
        115                 120                 125

Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys
    130                 135                 140

Asn
145
```

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 535 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

```
CTTAAGGTAA GTTCTTATCA AGTTTGTTCT TCTAATGTTT GATAGTTAAA GTATGTGTTA     60

TATTTGCTAA TTTTCTTACT CTAAAGGAAG TTAAAAATGA CGTCAAAATA AGCGTCGTAG    120

GAGGCGTAAT CGACGAGGTC AGTTGTGATG TTGTCTTCTA CTTTGCCGTG TTTAAGGCCG    180

ACTTCGACAG TAGCCAATGA GTCTAAATCT TCCCCTAAAG CTACAACGAC AAAACGGTAA    240

AAGGTTGTCG TGTTTATTGC CCAATAACAA ATATTTATGA TGATAACGGT CGTAACGACG    300

ATTTCTTCTT CCCCATAGGT ACCGATTCTC TCTTCTTCGA CTTCGACTTC GATCTAAGCA    360

ATTGGTTGTG AACACGCCAA GGGTGAACCA ACTTCGAAAC ATGAACCAAA CACCACTTTC    420

TCCAAAGAAG ATGTGAGGTT TCTGATCTCC ATAGCAACTT GTTACAACAT GAAGATAGAC    480

AAGAAACATG GTTAACCTTT TGATGACGTT GATCTGCGTC GGGCGTCCGA GATCT         535
```

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 538 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 77..514

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

```
GAATTCCATT CAAGAATAGT TCAAACAAGA AGATTACAAA CTATCAATTT CATACACAAT     60

ATAAACGATT AAAAGA ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA         109
                  Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu
                    1               5                  10

TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA       157
Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu
              15                  20                  25

GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT       205
Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp
          30                  35                  40

TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA       253
Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr
      45                  50                  55

AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT       301
```

-continued

```
Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala
 60                  65                  70                  75

AAA GAA GAA GGG GTA TCC ATG GCT AAG AGA GAA GAA GCT GAA GCT GAA      349
Lys Glu Glu Gly Val Ser Met Ala Lys Arg Glu Glu Ala Glu Ala Glu
                 80                  85                  90

GCT GAA AGA TTC GTT AAC CAA CAC TTG TGC GGT TCC CAC TTG GTT GAA      397
Ala Glu Arg Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu
             95                  100                 105

GCT TTG TAC TTG GTT TGT GGT GAA AGA GGT TTC TTC TAC ACT CCA AAG      445
Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys
         110                 115                 120

ACT AGA GGT ATC GTT GAA CAA TGT TGT ACT TCT ATC TGT TCT TTG TAC      493
Thr Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr
     125                 130                 135

CAA TTG GAA AAC TAC TGC AAC TAGACGCAGC CCGCAGGCTC TAGA               538
Gln Leu Glu Asn Tyr Cys Asn
140                 145
```

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 146 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

```
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
  1               5                  10                  15

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
             20                  25                  30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
         35                  40                  45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
     50                  55                  60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
 65                  70                  75                  80

Ser Met Ala Lys Arg Glu Glu Ala Glu Ala Glu Ala Glu Arg Phe Val
                 85                  90                  95

Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val
            100                 105                 110

Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr Arg Gly Ile Val
        115                 120                 125

Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr
    130                 135                 140

Cys Asn
145
```

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 538 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

```
CTTAAGGTAA GTTCTTATCA AGTTTGTTCT TCTAATGTTT GATAGTTAAA GTATGTGTTA     60
```

-continued

```
TATTTGCTAA TTTTCTTACT CTAAAGGAAG TTAAAAATGA CGTCAAAATA AGCGTCGTAG    120

GAGGCGTAAT CGACGAGGTC AGTTGTGATG TTGTCTTCTA CTTTGCCGTG TTTAAGGCCG    180

ACTTCGACAG TAGCCAATGA GTCTAAATCT TCCCCTAAAG CTACAACGAC AAAACGGTAA    240

AAGGTTGTCG TGTTTATTGC CCAATAACAA ATATTTATGA TGATAACGGT CGTAACGACG    300

ATTTCTTCTT CCCCATAGGT ACCGATTCTC TCTTCTTCGA CTTCGACTTC GACTTTCTAA    360

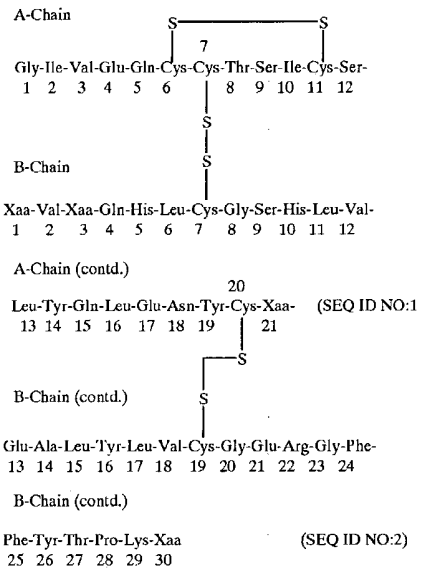
GCAATTGGTT GTGAACACGC CAAGGGTGAA CCAACTTCGA AACATGAACC AAACACCACT    420

TTCTCCAAAG AAGATGTGAG GTTTCTGATC TCCATAGCAA CTTGTTACAA CATGAAGATA    480

GACAAGAAAC ATGGTTAACC TTTTGATGAC GTTGATCTGC GTCGGGCGTC CGAGATCT      538
```

We claim:

1. An insulin derivative having the following sequence:



wherein

(a) Xaa at positions A21 and B3 are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys;

(b) Xaa at position B1 is Phe or is deleted;

(c) Xaa at position B30 is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; and

(d) the ε-amino group of Lys^{B29} is substituted with a lipophilic substituent having at least 10 carbon atoms;

wherein the insulin derivative is a Zn^{2+} complex and the Zn^{2+} complex of the insulin derivative is more water soluble than the insulin derivative without Zn^{2+}.

2. The insulin derivative according to claim **1**, wherein Xaa at position A21 is Asn.

3. The insulin derivative according to claim **2**, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

4. The insulin derivative according to claim **1**, wherein Xaa at position A21 is Ala, Asn, Gln, Gly or Ser.

5. The insulin derivative according to claim **4**, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

6. The insulin derivative according to claim **1**, wherein Xaa at position B1 is deleted.

7. The insulin derivative according to claim **6**, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

8. The insulin derivative according to claim **1**, wherein Xaa at position B1 is Phe.

9. The insulin derivative according to claim **8**, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

10. The insulin derivative according to claim **1**, wherein Xaa at position B3 is Asn, Asp, Gln or Thr.

11. The insulin derivative according to claim **10**, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

12. The insulin derivative according to claim **1**, wherein Xaa at position B30 is Ala or Thr.

13. The insulin derivative according to claim **12**, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

14. The insulin derivative according to claim **1**, wherein Xaa at position A21 is Ala, Asn, Gln, Gly or Ser, Xaa at position B3 is Asn, Asp, Gln or Thr, and Xaa at position B30 is Ala or Thr.

15. The insulin derivative according to claim **14**, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

16. The insulin derivative according to claim **1**, wherein Xaa at position A21 is Asn, Xaa at position B3 is Asn, Xaa at position B1 is Phe and Xaa at position B30 is Thr.

17. The insulin derivative according to claim **16**, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

18. The insulin derivative according to claim **1**, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

19. The insulin derivative according to claim **1** which is in the form of a hexamer.

20. The insulin derivative according to claim **19**, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

21. The insulin derivative according to claim **19**, wherein Xaa at position A21 is Asn, Xaa at position B1 is Phe, Xaa at position B3 is Asn, and Xaa at position B30 is Thr.

22. The insulin derivative according to claim **19**, wherein two zinc ions bind to the hexamer.

23. The insulin derivative according to claim **22**, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

24. The insulin derivative according to claim **19**, wherein three zinc ions bind to the hexamer.

25. The insulin derivative according to claim **24**, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

26. The insulin derivative according to claim **19**, wherein four zinc ions bind to the hexamer.

27. The insulin derivative according to claim **26**, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

28. A pharmaceutical composition which is an aqueous solution, comprising (a) an insulin derivative according to claim **1**, (b) an isotonic agent, (c) a preservative and (d) a buffer.

29. The pharmaceutical composition according to claim **28**, wherein the pH of the aqueous solution is in the range of 6.5–8.5.

30. The pharmaceutical composition according to claim 28, wherein the solubility of the insulin derivative exceeds 600 nmol/ml of the aqueous solution.

31. The pharmaceutical composition according to claim 28, further comprising an insulin or an insulin analogue which has a rapid onset of action.

32. The pharmaceutical composition according to claim 28, wherein Xaa at position A21 is Asn, Xaa at position B3 is Asn, Xaa at position B1 is Phe and Xaa at position B30 is Thr.

33. The pharmaceutical composition according to claim 28, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

34. The pharmaceutical composition according to claim 28, wherein the insulin derivative is in the form of a hexamer.

35. A method of treating diabetes in a patient in need of such a treatment, comprising administering to the patient a therapeutically effective amount of a pharmaceutical composition according to claim 28.

36. The insulin derivative of claim 1, wherein the lipophilic substituent is cyclohexylvaleroyl.

37. The insulin derivative of claim 1, wherein the lipophilic substituent is acyl-glutamyl wherein the acyl is a linear, saturated acyl having 6 to 24 carbon atoms.

38. The insulin derivative of claim 1, wherein the lipophilic substituent is lauroyl.

39. The insulin derivative of claim 1, wherein the lipophilic substituent is myristoyl.

40. The insulin derivative of claim 1, wherein the lipophilic substituent is palmitoyl.

41. The insulin derivative of claim 1, wherein the lipophilic substituent is 2-succinylamido myristic acid.

42. The insulin derivative of claim 1, wherein the lipophilic substituent is 2-succinylamido palmitic acid.

43. The insulin derivative of claim 1, wherein the lipophilic substituent is 2-succinylamidoethyloxy palmitic acid.

44. The insulin derivative of claim 1, wherein the lipophilic substituent is myristoyl-α-glutamyl.

45. The insulin derivative of claim 1, wherein the lipophilic substituent is myristoyl-α-glutamyl-glycyl.

46. The insulin derivative of claim 1, wherein the lipophilic substituent is choloyl.

47. The insulin derivative of claim 1, wherein the lipophilic substituent is 7-deoxycholoyl.

48. The insulin derivative of claim 1, wherein the lipophilic substituent is lithocholoyl.

49. The insulin derivative of claim 1, wherein the lipophilic substituent is lithocholoyl-glutamyl.

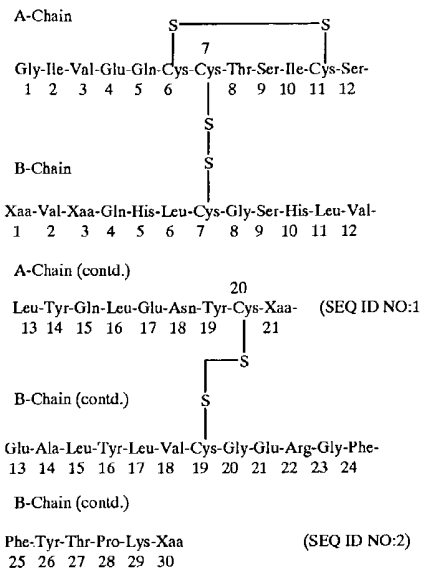
50. The insulin derivative of claim 1, wherein the lipophilic substituent is 4-benzoyl-phenylalanine.

51. The insulin derivative of claim 1, wherein the lipophilic substituent is L-thyroxyl.

52. The insulin derivative of claim 1, wherein the lipophilic substituent is suberoyl-D-thyroxine.

53. The insulin derivative of claim 1, wherein the lipophilic substituent is 3,3',5,5'-tetraiodothyroacetyl.

54. An insulin derivative having the following sequence:



(SEQ ID NO:1)

(SEQ ID NO:2)

wherein

(a) Xaa at positions A21 and B3 are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys;

(b) Xaa at position B1 is Phe or is deleted;

(c) Xaa at position B30 is deleted; and

(d) the ϵ-amino group of Lys^{B29} is substituted with a lipophilic substituent having at least 10 carbon atoms;

wherein the insulin derivative is a Zn^{2+} complex and the Zn^{2+} complex of the insulin derivative is more water soluble than the insulin derivative without Zn^{2+}.

55. The insulin derivative according to claim 54, wherein Xaa at position A21 is Ala, Asn, Gln, Gly or Ser.

56. The insulin derivative according to claim 55, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

57. The insulin derivative according to claim 54, wherein Xaa at position B1 is deleted.

58. The insulin derivative according to claim 57, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

59. The insulin derivative according to claim 54, wherein Xaa at position B1 is Phe.

60. The insulin derivative according to claim 59, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

61. The insulin derivative according to claim 54, wherein Xaa at position B3 is Asn, Asp, Gln or Thr.

62. The insulin derivative according to claim 61, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

63. The insulin derivative according to claim 54 wherein Xaa at position A21 is Ala, Asn, Gln, Gly or Ser, and Xaa at position B3 is Asn, Asp, Gln or Thr.

64. The insulin derivative according to claim 63, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

65. The insulin derivative according to claim 54, wherein Xaa at position A21 is Asn, Xaa at position B1 is Phe, and Xaa at position B3 is Asn.

66. The insulin derivative according to claim 65, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

67. The insulin derivative according to claim 54, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

68. The insulin derivative according to claim 54 which is in the form of a hexamer.

69. The insulin derivative according to claim 68, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

70. The insulin derivative according to claim 68, wherein Xaa at position A21 is Asn, Xaa at position B3 is Asn, and Xaa at position B1 is Phe.

71. The insulin derivative according to claim 68, wherein two zinc ions bind to the hexamer.

72. The insulin derivative according to claim 71, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

73. The insulin derivative according to claim 68, wherein three zinc ions bind to the hexamer.

74. The insulin derivative according to claim 73, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

75. The insulin derivative according to claim 68, wherein four zinc ions bind to the hexamer.

76. The insulin derivative according to claim 75, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

77. A pharmaceutical composition which is an aqueous solution, comprising (a) an insulin derivative according to claim 54, (b) an isotonic agent, (c) a preservative and (d) a buffer.

78. The pharmaceutical composition according to claim 77, wherein the pH of the aqueous solution is in the range of 6.5–8.5.

79. The pharmaceutical composition according to claim 77, wherein the solubility of the insulin derivative exceeds 600 nmol/ml of the aqueous solution.

80. The pharmaceutical composition according to claim 77, further comprising an insulin or an insulin analogue which has a rapid onset of action.

81. The pharmaceutical composition according to claim 77, wherein the insulin derivative is a Zn^{2+} complex.

82. The pharmaceutical composition according to claim 77, wherein Xaa at position A21 is Asn, Xaa at position B3 is Asn, and Xaa at position B1 is Phe.

83. The pharmaceutical composition according to claim 77, wherein the lipophilic substituent has from 12 to 24 carbon atoms.

84. The pharmaceutical composition according to claim 77, wherein the insulin derivative is in the form of a hexamer.

85. A method of treating diabetes in a patient in need of such a treatment, comprising administering to the patient a therapeutically effective amount of a pharmaceutical composition according to claim 77.

86. The insulin derivative of claim 54, wherein the lipophilic substituent is cyclohexylvaleroyl.

87. The insulin derivative of claim 54, wherein the lipophilic substituent is acyl-glutamyl wherein the acyl is a linear, saturated acyl having 6 to 24 carbon atoms.

88. The insulin derivative of claim 54, wherein the lipophilic substituent is lauroyl.

89. The insulin derivative of claim 54, wherein the lipophilic substituent is myristoyl.

90. The insulin derivative of claim 54, wherein the lipophilic substituent is palmitoyl.

91. The insulin derivative of claim 54, wherein the lipophilic substituent is 2-succinylamido myristic acid.

92. The insulin derivative of claim 54, wherein the lipophilic substituent is 2-succinylamido palmitic acid.

93. The insulin derivative of claim 54, wherein the lipophilic substituent is 2-succinylamidoethyloxy palmitic acid.

94. The insulin derivative of claim 54, wherein the lipophilic substituent is myristoyl-α-glutamyl.

95. The insulin derivative of claim 54, wherein the lipophilic substituent is myristoyl-α-glutamyl-glycyl.

96. The insulin derivative of claim 54, wherein the lipophilic substituent is choloyl.

97. The insulin derivative of claim 54, wherein the lipophilic substituent is 7-deoxycholoyl.

98. The insulin derivative of claim 54, wherein the lipophilic substituent is lithocholoyl.

99. The insulin derivative of claim 54, wherein the lipophilic substituent is lithocholoyl-glutamyl.

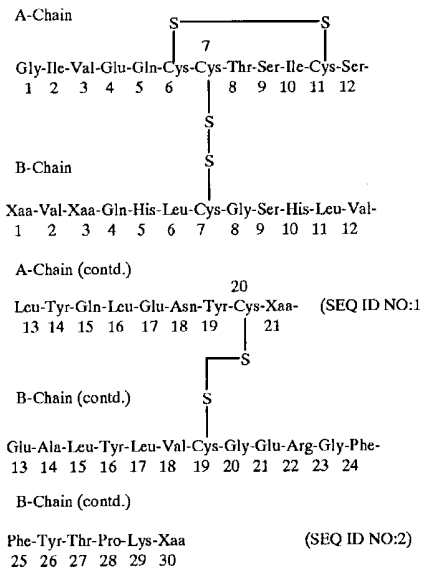
100. The insulin derivative of claim 54, wherein the lipophilic substituent is 4-benzoyl-phenylalanine.

101. The insulin derivative of claim 54, wherein the lipophilic substituent is L-thyroxyl.

102. The insulin derivative of claim 54, wherein the lipophilic substituent is suberoyl-D-thyroxine.

103. The insulin derivative of claim 54, wherein the lipophilic substituent is 3,3',5,5'-tetraiodothyroacetyl.

104. An insulin derivative having the following sequence:



wherein

(a) Xaa at positions A21 and B3 are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys;

(b) Xaa at position B1 is Phe or is deleted;

(c) Xaa at position B30 is any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys; and

(d) the ε-amino group of Lys^{B29} is substituted with a lipophilic substituent having at least 10 carbon atoms;

wherein the lipophilic substituent is benzoyl, phenylacetyl, cyclohexylacetyl, 3,5-diiodotyrosyl or cyclohexylpropionyl and the insulin derivative is a Zn^{2+} complex and the Zn^{2+} complex of the insulin derivative is more water soluble than the insulin derivative without Zn^{2+}.

105. The insulin derivative of claim 104, wherein the lipophilic substituent is benzoyl.

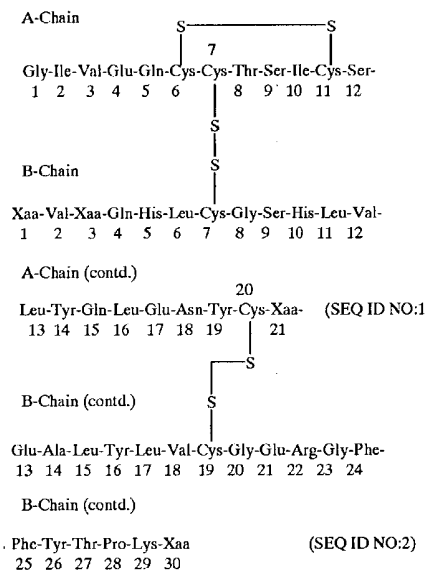
106. The insulin derivative of claim 104, wherein the lipophilic substituent is phenylacetyl.

107. The insulin derivative of claim 104, wherein the lipophilic substituent is cyclohexylacetyl.

108. The insulin derivative of claim 104, wherein the lipophilic substituent is 3,5-diiodotyrosyl.

109. The insulin derivative of claim 104, wherein the lipophilic substituent is cyclohexylpropionyl.

110. An insulin derivative having the following sequence:



wherein

(a) Xaa at positions A21 and B3 are, independently, any amino acid residue which can be coded for by the genetic code except Lys, Arg and Cys;

(b) Xaa at position B1 is Phe or is deleted;

(c) Xaa at position B30 is deleted; and

(d) the ϵ-amino group of Lys^{B29} is substituted with a lipophilic substituent having at least 10 carbon atoms;

wherein the lipophilic substituent is benzoyl, phenylacetyl, cyclohexylacetyl, 3,5-diidotyrosyl or cyclohexylpropionyl and the insulin derivative is a Zn^{2+} complex and the Zn^{2+} complex of the insulin derivative is more water soluble than the insulin derivative without Zn^{2+}.

111. The insulin derivative of claim **110**, wherein the lipophilic substituent is benzoyl.

112. The insulin derivative of claim **110**, wherein the lipophilic substituent is phenylacetyl.

113. The insulin derivative of claim **110**, wherein the lipophilic substituent is cyclohexylacetyl.

114. The insulin derivative of claim **110**, wherein the lipophilic substituent is 3,5-diidotyrosyl.

115. The insulin derivative of claim **110**, wherein the lipophilic substituent is cyclohexylpropionyl.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,011,007
DATED : January 4, 2000
INVENTOR(S) : Havelund et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,
Item [62], **Related U.S. Application Data**, please delete "Continuation-in-part of application No. 08/400,256, March 8, 1995, U.S. Pat. No. 5,750,497, which is a continuation-in-part of application No. 08/190,829, filed as application No. PCT/DK94/00347, Sep. 16, 1994, abandoned"
and insert
-- Continuation-in-part of application No. 08/400,256, filed March 8, 1995, U.S. Pat. No. 5,750,497, which is a continuation-in-part of application No. 08/190,829, filed Feb. 2, 1994, now abandoned, and application No. PCT/DK94/00347, filed Sep. 16, 1994, now abandoned --.

Column 1,
Line 7, please delete "5,750,997" and insert -- 5,750,497 --.

Signed and Sealed this

Twenty-sixth Day of November, 2002

Attest:

Attesting Officer

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

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